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# Factors affecting expression of soybean sudden death syndrome: Flooding, oxygen level, and ethylene hormone

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**Factors affecting expression of soybean sudden death syndrome: Flooding, oxygen level, and ethylene hormone**

by

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## DEDICATION

This dissertation is dedicated to my father, Eng. **Aly**, and my mother, **Salwa**, who have always encouraged and supported me throughout my life, and to my brother, Dr. **Hossam** who supported me scientifically. Also, this dissertation is dedicated to my wife **Hend** who stayed with me shoulder to shoulder and lived with me the entire rise and fall of my graduate school life until we did it. And finally, to my three little angels **Logayen**, **Lamar**, and **Lara** for making me feel happy. Thanks so much.

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## ABSTRACT

Soybean [*Glycine max* (L.) Merrill] sudden death syndrome (SDS), caused by the soilborne fungus *Fusarium virguiforme* (*Fv*), is a very damaging disease in North and South America, with average yield losses in the Unites States estimated at 190 million dollars annually between 1999 and 2004. Major SDS outbreaks have coincided with years of extreme flooding, such as 1993, 2008, and 2010, but there is no information about how and why excessive soil moisture is associated with severe SDS. In this study, the first objective was to investigate the effect of different flood regimes on the development of SDS under greenhouse conditions. Flooding was found to influence SDS disease severity and *Fv* population density in soil, but the overall effect on SDS development depended on duration of the flooding period. Short-term flooding, such as 3 days of continuous flooding or repeated flooding periods of 8 h a week for 3 weeks, generally predisposed soybean seedlings to SDS, whereas continuous flooding for 5 or 7 days resulted in lower SDS severity and lower *Fv* population in soil, compared to non-flooded controls.

Flooding conditions cause a decrease in oxygen levels and build-up of carbon dioxide and toxic compounds in the root zone. The second objective of this study was to test the effect of low oxygen levels, similar to those that may occur during flooding conditions, on the soybean-*Fv* interaction. A hydroponic system was established in a growth chamber where soybean seedlings were exposed to different oxygen levels and tested for the expression of soybean defense-related genes and *Fv* virulence genes using qPCR. Soybean seedlings exposed to anaerobic conditions for 12 hours exhibited down-regulation of key defense-related genes, including laccase and pathogenesis related

proteins, All *Fv* virulence genes tested, including pectate lyase, pisatin demethylase, and FvTox1also showed down-regulation in soybean roots exposed to anaerobic conditions for 12 hours.

Ethylene is a gaseous hormone involved in multiple plant growth and developmental processes, as well as response to biotic and abiotic stresses. The third objective of this study was to understand the role of ethylene hormone in soybean-Fv interaction. Soybean plants treated with ethephon (ethylene releasing compound) developed less severe SDS symptoms compared to water-treated seedlings, whereas those treated with cobalt chloride (ethylene biosynthesis suppressor) or 1-MCP (ethylene perception suppressor) showed the same or higher SDS foliar severity compared to the water treated control. Ethephon application also resulted in activation of genes involved in ethylene biosynthesis, and genes involved in soybean defense responses, such as phenylpropanoid pathway, pathogenesis related proteins and transcription factors. Cobalt chloride and 1-MCP treatments had little or no effect on these genes. Moreover, ethephon had an inhibitory effect on in-vitro growth of *Fv* on potato dextrose agar.

The fourth objective of this study was to determine the optimum application timing of ethephon to suppress SDS development in greenhouse and field conditions. In the greenhouse, all ethephon applications significantly reduced SDS foliar symptom severity by 50-60% compared to the untreated control in susceptible cultivar Williams82. In field studies conducted in 2015, ethephon application at planting (in-furrow) or after plant emergence (VE growth stage) reduced SDS foliar severity compared to the untreated control. Plots that received ethephon at VE growth stage showed a 15% increase ($P$=0.08) in yield compared to untreated plots.

Overall, the results of this research enhance understanding of how flooding and the accompanied anaerobic conditions affect SDS development, and demonstrate that the ethylene-signaling pathway plays an important role in resistance against SDS. Finally, our findings suggest that the use of plant defense inducers, such as ethephon, can suppress SDS and encourage future investigation on their use for SDS management under filed conditions.

# CHAPTER 1.

# GENERAL INTRODUCTION

## Dissertation Organization

This dissertation is organized into five chapters. The first chapter provides a literature review of the history of soybean, the economic impact of flooding and sudden death syndrome (SDS) on soybean production, and the role of ethylene hormone on plant disease. The second chapter describes a greenhouse study on the effect of flooding duration on SDS development and a growth chamber experiment on the effect of different oxygen levels on *Fusarium virguliforme* (*Fv*) virulence genes and soybean defense-related genes. The third chapter includes a set of greenhouse and growth chamber experiments to test the effect of ethylene induction (using ethephon, an ethylene releasing compound), and ethylene suppression (using cobalt chloride) on SDS development and the *in-vitro* growth of *Fv*. The fourth chapter describes a set of field and greenhouse experiments to test the effect of ethephon on SDS development and soybean yield. Finally, the fifth chapter covers the general conclusions of the research presented in this dissertation.

## Literature Review

### The importance of soybean crop production

Soybean [*Glycine max* (Merr.) L.], is one of the most important crops around the world. It was first domesticated in China around 1100 BC, and since then soybean production has spread all over the world as a valuable source of vegetable protein and oil. Soybean was first planted in

North America in 1765, in the then-British colony of Georgia (Hartman et al. 2011). At the beginning of the 20th century, the United States Department of Agriculture encouraged farmers to plant soybean, and the U.S is one of the world's leaders in soybean production, with about 32% of the world's soybean production. In terms of planting area and production, soybean is ranked the second most important crop in the U.S (corn is ranked first), with 72 million acres planted and 2.85 billion bushels produced each year (United Soybean Board). Iowa and Illinois are the largest soybean producing states in the United States, with total production of 466.11 and 416.42 million bushels, respectively (2011 harvest; National Agriculture Statistics Services, 2012). Despite the large soybean production in the U.S, yield loss from abiotic and biotic stresses are still high and cause economic problems for soybean growers and the soybean industry (Wrather and Koenning 2009; Wrather et al. 2010; Board and Kahlon 2011).

Abiotic stresses such as drought, flooding, salinity, soil fertility, and extreme temperatures have detrimental effects on soybean growth and development, and account for more than 50% yield losses worldwide (Bray et al. 2000). For instance, drought stress during vegetative or seed development stages (Fehr et al. 1971) results in reduced seed number and seed size, respectively (Vieira et al. 1992; Brevedan and Egli 2003). High temperature during seed filling can reduce seed germination and vigor under field conditions (Egli et al. 2005). Salinity stress reduces soybean plant chlorophyll, and thereby has negative impacts on yield. Salinity stress also affects seed quality and is associated with lower oil and protein content in seeds (Ghassemi-golezani and Taifeh-noori 2009). Flooding at early vegetative or reproductive stages may cause yield losses up to 43% and 56%, respectively (Vantoai et al. 2001).

Biotic stresses, such as pathogens, insects, and weeds are additional damaging factors for soybean production. Yield reduction due to disease is estimated to be 11% per year worldwide

(Hartman et al. 2011). In the United States, soybean yield suppression due to diseases averaged approximately 484.4 million bushels from 2006 to 2009 (Koenning and Wrather 2010). The most damaging pathogens are soybean cyst nematode (SCN), which is ranked first among the diseases that suppress soybean yield, followed by Phytophthora root rot (*Phytophthora sojae*), and sudden death syndrome (*Fusarium virguliforme*) (Wrather and Koenning, 2009).

**Sudden death syndrome (SDS)**

Soybean sudden death syndrome (SDS), caused by *Fusarium virguliforme, F. brasiliense, F. cuneirostrum,* or *F. tucumaniae* (Aoki et al. 2005, 2003), is one of the most damaging diseases to the soybean production worldwide. The disease has been reported from all major soybean producing countries. In the U.S, the disease was first observed in Arkansas in 1971, but it has since spread to all major soybean growing states (Roy et al. 1997). During the 1980s the disease was reported in Tennessee, Mississippi, Illinois, Indiana, and Missouri (Roy et al. 1997; Rosenbrock, 1988). In the 1990s, SDS was reported in Kansas (Jardine and Rupe, 1993) and Iowa (Yang and Rizvi, 1994). Since 2000, SDS has been reported in Minnesota (Kurle et al. 2003), Wisconsin (Bernstein et al. 2006), Nebraska (Ziems et al. 2006), Michigan (Chilvers and Brown-Rytlewski, 2010), and South Dakota (Tende et al. 2014). Outside the U.S., the disease was reported in Argentina (Ploper, 1993), Brazil (Nakajima et al. 1993), Canada (Anderson and Tenuta 1998), Paraguay (Yorinori, 1999), Bolivia (Yorinori, 2002), Uruguay (Ploper et al. 2003), and South Africa (Tewoldemedhin et al. 2014).

*F. virguliforme* is a soil-borne pathogen and it infects the roots, resulting in root rot and reduction in root biomass. Aboveground symptoms of Fv infection are interveinal chlorosis and necrosis, premature defoliation, and pod abortion as a result of phytotoxin translocation from the

roots (Jin et al. 1996). Root infection can happen soon after planting, while foliar symptoms may not develop until soybean reproductive stages (Hartman et al. 2015). Yield loss is highly dependent on cultivar, growth stage, and environmental conditions (Roy et al. 1997).

SDS is ranked among the top ten disease that suppress soybean yield in the U.S., with average yield losses ranged from 3.7 to 75.7 million bushels, estimated from 28 states during the period of 1996 to 2007 (Wrather and Koenning 2009). Resistant cultivars and certain cultural practices (tillage, improvement of soil drainage, planting date, and crop rotation) are currently used by growers to manage SDS (Wrather et al. 1995; Rupe et al. 1997). However, disease severity is highly dependent on environmental conditions, such as soil moisture, temperature, and soil variables such as pH, and organic matter (Gongora-Canul and Leandro 2011; Scherm and Yang 1996; Scherm et al. 1998), adding more difficulty to the disease management.

**Geographic distribution of SDS causal agents**

SDS is caused by four morphologically distinct *Fusarium* species: *F. brasiliense, F. cuneirostrum, F. tucumaniae,* and *F. virguliforme.* In South America, all four species are present and induce SDS symptoms on soybeans. In Brazil, *F. brasiliense* and *F. cuneirostrum* are responsible for causing soybean SDS. *F. virguliforme* and *F. tucumaniae* cause SDS in Argentina. *F. virguliforme* is the only known causal agent of SDS in North America (Aoki et al. 2005).

***Fusarium virguliforme* classification and morphology**

**Classification**. *F. virguliforme* was formerly known as *F. solani* (Mart.) Sacc. f. sp. *glycines*, but is now classified within the *F. solani* species complex (section *Martiella*) (Aoki et

al., 2005). Phylogenetic studies based on nuclear 28S rDNA, ribosomal ITS region and ER-1α gene for the *F. solani* species complex showed that *F. virguliforme* is closely related to *F. tucumaniae* and *F. solani* f. sp. *phaseoli* (the root-rot pathogen of *Phaseolus vulgaris* L.) within the South American clade of the *F. solani* species complex. Thus, all three species may have evolutionary origins in the southern hemisphere (O'Donnell 2000; Aoki et al. 2003). However, the two SDS species do not form a monophyletic group, which suggests that their pathogenicity to soybean evolved convergently, or perhaps the most recent common ancestor of *F. solani* f. sp. *phaseoli* lost its pathogenicity to soybean (Njiti et al. 1997).

**Morphology.** *F. virguliforme* grows slowly on PDA with radial mycelial growth rate of 1.3-1.7 mm per day at 20°C in the dark (Aoki et al. 2003). The blue pigmentation associated with the SDS causal agent is invariably produced by the pathogen in the culture, although the colonies exhibit variation in pigmentation based on sporulation amount. *Fusarium virguliforme* macroconidia are formed from monophialides on either simple or branched conidiophores, and have from two to five septa, while microconidia are rarely formed. On PDA media, macroconidia dimensions range from 30 to 65 µm long by 6 to 8 µm wide, and on modified Bilay's media the average dimensions are 50.5 µm long by 4.6 µm wide. Chlamydospores may be formed in individual macroconidia or in pairs, either terminally or inhyphae. Sporulation occurs abundantly and rapidly on SNA and PDA media, with greenish to bluish pigmentation under daylight. The septate, falcate aerial macroconidia with a foot cell on tall slender conidiophores, can be used to distinguish *F. virguliforme* from other species in the *F. solani* complex (Roy et al. 1997; Aoki et al. 2005). Only infertile sexual structures (perithecia) were produced from crosses between *F. virguliforme* and *F. tucumaniae.* Existing reports suggest that *F. virguliforme* reproduces

asexually, and that this is possibly due to the absence of the positive mating type within the U.S. populations, or that sexual reproduction requires different environmental conditions (Covert et al. 2007).

**Symptoms and signs**

SDS is a disease of two stages. The first stage starts below ground when *F. virguliforme* colonizes the roots, causing root rot and reduction in the root biomass, followed by the second stage, which is characterized by upward movement of fungal toxins through the vascular system, which causes the aboveground symptoms. Root symptoms are characterized by internal grayish to reddish brown discoloration near the pith, which may extend from the taproot up into the stem for several nodes, while the pith remains white. Severe leaf symptoms are associated with internal root discoloration, pronounced necrosis of the taproot and lateral roots, substantial root mass reduction, and premature plant death (Roy et al. 1997). Root infection is highly dependent on environmental conditions, and cool (15-17°C), wet soil increases disease severity in early soybean planting. Under controlled environment conditions, root infection can happen soon after planting (Scherm and Yang 1996), whereas under field conditions, infection can take up to 18 days after planting (Gao et al. 2006).

During soybean reproductive stages, foliar symptoms first appear as irregular scattered chlorotic spots, then progress to interveinal chlorosis and necrosis, premature defoliation, and finally flowers and pods abortion. The upper leaves always defoliate faster than the lower leaves, as well as the upper flowers and pods abort first. Foliar symptoms develop rapidly during the reproductive stages R2 to R5, but then increase more slowly in later soybean growth stages (Roy et al. 1997). The relationship between root symptoms and foliar symptoms has been studied under

controlled and field conditions. Working in a controlled environment, Scherm and Yang (1996) found no close correlation between root and foliar disease severity. One explanation for this phenomenon is that foliar and root disease symptoms require different environmental conditions to be expressed. However, a field study by Luo et al. (1999) showed a correlation between yield loss, foliar disease index, and root colonization, which was identified by analysis of pathogen temporal dynamics. A study by Navi and Yang (2008) showed that *Fv* colonization of xylem root tissue is critical for SDS foliar symptoms expression. The authors observed fungal structures in the xylem and phloem tissues of plants with SDS foliar symptoms, while plants with no foliar symptoms showed fungal structures in phloem tissue only. This study also reported a positive relationship between disease severity index and taproot discoloration.

**Disease cycle and favorable environmental condition**

Chlamydospores are considered to be the primary inoculum source of *Fv*. The *Fv* macroconidia are converted to chlamydospores in a soil solution extract and were observed in degraded, sloughed cortical tissue (Melgar et al., 1994; Roy et al., 1997). Chlamydospores can overwinter in crop residues, in SCN cysts, or in the soil. Early in the spring, when soil is cool (15-17°C) and wet, chlamydospores germinate to produce hyphae that penetrate the root either near the root-cap zone or at the bases of root hairs. Inside the root, hyphae grow both intercellularly and intracellularly. After the fungus colonizes the root and causes necrosis, secondary metabolites phytotoxin are released and transported through the root vascular system to the leaves, resulted in expression of the foliar symptoms starting with chlorotic mottling that progresses to interveinal chlorosis, necrosis, and defoliation (Roy et al. 1997).

**SDS disease management**

The disease progress of SDS is highly dependent on several biotic and abiotic factors, such as environmental conditions, soil moisture and temperature, cultivar, growth stage at infection time, planting date, soybean cyst nematode (SCN), and interactions among these factors (Wrather et al. 1995; Rupe, 1995; Scherm and Yang, 1996; Sanogo and Yang 2000), thus multiple management tools should be used to control SDS rather than a single strategy. Currently, disease management strategies such as planting resistant varieties, practicing tillage and crop rotation, and improving soil drainage are important means of managing SDS damage and yield loss.

**Planting date.** Early planting is often associated with increased SDS disease severity. Cool (15°C), wet soil early in the growing season, followed by intermediate temperatures (22-24°C) during soybean reproductive development, are favorable environmental conditions for SDS symptom development (Scherm and Yang 1996). Mid-May plantings showed greater SDS symptoms in most cultivars as compared to planting in late June or early July (Wrather et al. 1995; Hershman et al. 1990). Late planting can allow the soil to be more dry and warm, which is unfavorable for *Fv* germination and may reduce SDS development. Thus, late planting is preferred to reduce the SDS epidemics that happen early in the growing season, especially when the soil is wet and cool. However, growers do not prefer this strategy as it reduces yield potential due to the insufficient day length for soybean to reach full maturity.

**Crop rotation.** Soybean-corn rotation has been used as a management tool by Midwestern soybean growers to increase crop yields (Pedersen and Lauer 2004). However, a recent study found that SDS severity was higher in rotation plots than in soybean monoculture plots (Xing and

Westphal 2009). In addition, a study in Iowa suggested that soil amended with crop residues such as corn kernels and roots resulted in high *Fv* population densities under greenhouse and field conditions (Navi and Yang 2016). Thus, corn-soybean rotation is not recommended to suppress SDS. Alternatively, Rupe et al (1997) found soybean rotation with sorghum or wheat significantly reduced *Fusarium virguliforme* and *Heterodera glycines* (SCN) population densities in soil. Furthermore, 3- and 4-year crop rotation study in Iowa using crops such as oat, red clover, and alfalfa showed less SDS severity and higher yield compared to 2-years soybean-corn rotation study (Leandro et al. 2012). An explanation for the inconsistent results of using crop rotation to reduce SDS is that *Fusarium virguliforme* can persist in unfavorable environmental conditions by forming thick- walled chlamydospores that can survive in soil for many years even in the presence of non-host plants (Roy et al. 1997). Also, a greenhouse study conducted by Kolander et al. (2010) showed that *Fv* was able to induce SDS root rot and foliar symptoms in alfalfa (*Medicago sativa*), pinto bean and navy bean (*Phaseolus vulgaris*), white clover (*Trifolium repens*), red clover (*T. pretense*), pea (*Pisum sativum*), and Canadian milk vetch (*Astragalus canadensis*). Crops selected for use in long-term crop rotations should be carefully tested against *Fv* prior to use in rotation studies.

**Tillage.** Inconsistent results for SDS severity were obtained by using tillage as a management tool. Higher SDS symptoms were observed in no-till fields than disk-till or ridge-till (Wrather et al. 1995). In addition, subsoil tillage significantly reduced SDS foliar symptoms, compared to no-till plantings, probably due to greater soil porosity and less soil moisture (Vick et al. 2003). However, a study in Indiana showed that more SCN eggs and higher SDS symptoms severity were observed under long term till than in no-till system (Westphal et al. 2008; 2009).

**Resistant cultivars.** Disease management using resistant cultivars is the most effective management tool against SDS, yet challenges still exist in getting plant lines with complete SDS resistance, probably due to the quantitative nature of SDS resistance and the effect of environmental conditions on symptom expression (Hnetkovsky et al. 1996; Roy et al. 1997; Nijiti et al. 2001; Neto et al. 2007). Plant breeders developed restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), microsatellite and amplified fragment length polymorphism (AFLP) molecular markers to identify and locate quantitative treat loci (QTLs) associated with SDS resistance. Initially, SDS resistance was thought to be controlled by single dominant gene *Rfs* (Stephens et al. 1993). However, a cross between the soybean cultivars Essex and Forest showed that SDS resistance controlled by several QTLs (Kassem et al. 2006). Recently, 12 *qRfs* loci on nine linkage groups responsible for SDS resistance were reported in a population of recombinant inbred lines (RIL) developed from crosses between Forest × Essex, Pyramid × Douglas, and Flyer × Hartwig (Iqbal et al., 2001; Njiti et al., 2002; Kazi et al., 2007). However, separate loci confer resistance to root infection, leaf scorching, and *Heterodera glycines,* resulting in different responses among cultivars to SDS (Njiti et al. 2002; Kazi et al. 2007). Furthermore, increased expression of candidate genes that are involved in defense, metabolism, cell structure and transport were observed in recombinant inbred line (RIL) 23, relative to expression in Forest and Essex soybean genotypes, which suggests that the QTLs for SDS resistance serve to delay symptoms or confer resistance by maintaining or increasing specific genes after infection (Iqbal et al. 2002).

**Soybean cyst nematode (SCN) management.** Soybean cyst nematode is a major pathogen for soybean and can cause significant yield reduction (Wrather and Koenning 2009), therefore reducing SCN population densities through rotation with non-host crops, chemical control, and using of SCN resistant varieties are important management tools to limit SDS and yield loss (Niblack and Tylka 2008). Initial studies on interaction between *Heterodera glycines* and *Fusarium virguliforme* reported that the presence of SCN increases SDS symptoms severity (Roy et al. 1989; Mclean and Lawrence 1993). A field study by McLean and Lawrence (1993) showed more severe SDS foliar symptoms in seedlings infested with both *F. virguliforme* and SCN than in seedlings inoculated only with *F. virguliforme*. In contrast, other studies have reported no or weak relationships between SCN and SDS foliar severity. In Kentucky, Hershman et al (1990) observed no correlation between cyst population densities and the area under the SDS disease progress curve (AUDPC). After studying soil variables associated with SDS in Iowa, Scherm et al. (1998) reported a positive but weak correlation between cyst counts and SDS disease severity. Gao et al. (2006) found that SCN population density did not increase SDS foliar symptoms or affect root colonization by the fungus, and that high *Fv* inoculum levels suppressed *H. glycines* reproduction.

## The effect of flooding on crop production

Flooding is one of the most damaging abiotic stresses for crop production worldwide and is always accompanied by huge economic losses. Due to the effect of climate change, flooding frequency has increased during the last decades, resulting in huge damages to agricultural production in many areas around the world (Bailey-Serres et al. 2012). Flooding can have direct impacts on crop productivity or indirectly affect production by changing soil quality (Bailey-

Serres et al. 2012). For example, in Europe, flooding due to increased summertime rainfall caused significant agriculture economic losses (Olesen et al. 2011). Also, lowland rice fields in Asia are more vulnerable to seasonal flash flooding and deep water flooding that may cause death of the entire plant (Ahmed et al. 2013).

In the U.S., 16% of the agricultural soils were affected by flooding due to heavy rainfall, poor drainage system, or high soil clay content as reported by Boyer (1982). In the period from 2000 to 2011, flooding was ranked the second most damaging abiotic stress to crop production, with damages to homes, business, and agriculture estimated to be billions of dollars. In 2011 flooding and drought accounted for more than 70% of the reduction in harvests worldwide (Bailey-Serres et al. 2012). In 1993, the central United States experienced flooding along the Mississippi River due to exceptionally heavy rainfall. Approximately 1.1 ha were flooded in Iowa, planting was delayed, 0.5 million ha of (corn, soybean, and oats) could not be harvested, and more than 0.2 million ha of corn was not planted (Munkvold 1995). Financial losses from the 1993 floods totaled billions of dollars.

**The effect of flooding on soybean production**

Soybean is very sensitive to flooding: 3 days of continuous flooding at the early vegetative growth stages can cause death or significant yield reduction (Sullivan et al. 2001; Linkemer et al. 1998). The lack of oxygen supply leads to symptoms like leaf chlorosis, reduced stem and root elongation, disruption of photosynthesis, stomatal closure and low transpiration rate, reduced nitrogen fixation, loss of membrane integrity, and reduced yield (Kozlowski, 1984; Oosterhuis et al. 1990; Linkemer et al. 1998). Flooding damage is highly dependent on soybean growth stage, cultivar, flooding duration, soil temperature, and the condition of flooding water. A greenhouse

study by Linkemer (1998) showed that early vegetative and reproductive stages are more sensitive to flooding damage than other stages. In that study flooding for more than 2 days at the V4 and R2 growth stages reduced yield by 18% and 26%, respectively (Scott et al. 1989). Also, a field study of 84 soybean cultivars in Ohio showed that flooding for 4 weeks at R1 to R2 stages reduced the average yield by 25% compared to the non-flooded controls (VanToai et al. 1994). Soybean is sensitive to flooding damage, although some genotypes are more tolerant than others, based on the physiological responses. Oosterhuis et al (1990) found that the cultivar Forrest is more tolerant to excess water, whereas Essex is less tolerant. Van Toai et al (2001) identified a single QTL from the parent line Archer that was significantly associated with flooding tolerance. Shannon et al. (2005) reported 40% yield reduction in flood tolerant cultivars versus 80% reduction in the sensitive group when flooding was imposed at the R1 stage.

**Soybean adaptation to flooding stress**

Despite the fact that soybean is sensitive to flooding damage, it has been shown that soybean can tolerate anaerobic conditions through morphological and physiological adaptation (Valliyodan et al. 2014). One of the primary responses to the anaerobic conditions is the accumulation of ethylene in soil and the submerged plant parts (Sairam et al. 2008), which in turn induces the formation of aerenchyma tissue, which arises by cell separation, differential expansion, or programmed cell death (Seago et al. 2005). Aerenchyma is a soft tissue that facilitates the diffusion of oxygen from the aerated shoots to the roots and the surrounding rhizosphere soil (Jackson and Armstrong 1999). Also, ethylene can induce the formation of adventitious roots, close to the soil surface, which emerge from the hypocotyl and functionally

replace the old root system. Adventitious roots can reduce the effect of anaerbiosis by increasing the exposed area of the roots to the air (Bailey-Serres et al. 2012).

**Soil anaerobiosis**

During normal conditions the soil oxygen concentration is equivalent to that of the atmosphere, but when soil is flooded, the air inside soil pores is replaced by water. Because oxygen diffuses more slowly into water than in air ($10^4$ time slower), the demand for oxygen from root and microorganism respiration exceeds the supply from the atmosphere. One study showed that the soil oxygen level declined from 21 to 0 kPa within 1-1.5 day, and that $CO_2$ levels rose simultaneously, creating an anaerobic environment around the root zone (Pezeshki 2001; Greenway et al. 2006). Facultative anaerobes will take advantage of these conditions, consume the available oxygen in the soil solution, and accumulate phytotoxic compounds (e.g., sulfides, $Mn^{+2}$, $Fe^{+2}$, and reduced forms of nitrate) that cause injuries to root metabolism (Laanbroek 1990; Pezeshki 2001). Consequently, water and nutrient uptake by roots will be inhibited, resulting in chlorotic, stunted plants and reduced yield (Linkemer et al. 1998).

**Flooding and plant diseases**

Exposure of plants to environmental stress such as flooding, drought, and anaerobic conditions prior to infection may induce plant disease by affecting host susceptibility, pathogen virulence, or both (Schoeneweiss 1975). For example, saturated soils caused by flooding, excessive rainfall, or poor drainage may increase or decrease the disease incidence and severity of plant diseases. Although, this will depend on the ability of soilborne pathogens to survive anaerobic conditions and successfully infect the host roots, the response of the plant host to

anaerobic conditions, and the activity of antagonistic microorganisms (Drew and Lynch 1980). High soil moisture content is important for root rot diseases caused by *Phytophthora*, *Pythium,* and *Fusarium* species (Kirkpatrick et al. 2006b; Yanar et al. 1997). For instance, weekly and biweekly application of flooding for 48 hours for 3 months increased the disease severity of root rot of blue berries caused by *Phytophthora cinnamomi*, compared to monthly flooding or no flooding applications (De Silva et al. 1999). Inoculation of tomato seedlings with zoospores of *Phytopthora parasitica* after imposing plant water stress increased disease severity of Phytophthora root rot (Ristaino and Duniway 1991). Likewise, Kuan and Erwin (1980) showed that soil saturation increased the susceptibility of alfalfa to *Phytophthora megasperma* f. sp. *medicaginis* by increasing root exudation, which attracts the zoospores.

Conversely, several studies have shown that soil flooding can be used as a management tool to control soilborne pathogens and decrease the severity of root rot diseases in many crops, probably due to the effect of anaerobic environment and the activity of the antagonistic microorganisms (Momma 2008). For example, Ioannou et al. (1977) reported that 40 days of continuous flooding reduced microsclerotia production of *Verticillium dahliae,* and Niem et al. (2013) showed that 18 weeks of soil flooding at 20°C reduced viability of *Sclerotinia sclerotiorum* sclerotia. Disease severity of Verticillium wilt of Chile pepper was greater under no flooding conditions compared to periodic flooding (Sanogo et al. 2008). Other studies showed that application of organic amendments, followed by irrigation and tarping to create an anaerobic environment, is a good management tool to control soil-borne pathogens including *Rhizoctonia solani*, *Fusarium oxysporum* f. sp.*lycopersici*, *Verticillium dahliae,* and *Ralstonia solanacearum* (Huang et al. 2015; Blok et al. 2000; Momma 2008).

**The effect of flooding and anaerobic conditions on soybean diseases**

Flooding and the accompanying anaerobic conditions can cause disease by negatively affecting soybean energy metabolism and physiological performance (Oosterhuis et al. 1990) which in turn make the host more vulnerable to infection, especially if these conditions are favorable for the pathogen (Kirkpatrick et al. 2006a). For instance, excessive wet soil creates ideal environmental conditions for many soilborne diseases such as Phytophthora root rot caused by *P. sojae*, Rhizoctonia root rot casued by *Rhizoctonia solani* , and Pythium damping-off caused by *Pythium ultimum* (Kirkpatrick et al. 2006 a,b; Dorrance et al. 2003). Soybean diseases are typically worse during flooding years; e.g., Iowa soybean production was greatly affected by 1993 floods along the Mississippi River, and the epidemics of seedling, foliar, and root diseases were severe (Munkvold 1995).

Transcriptome and proteomic analyses of flooded and low oxygen stressed soybean seedlings showed transcriptional and post-transcriptional changes, which are involved in causing injury to soybean seedlings. For instance, Komatsu et al. (2010) reported that lignification was suppressed in flooded soybean roots due to down regulation of proteins such as lipoxygenase, a germin-like protein precursor, glycoprotein precursor, and superoxide dismutase, which were associated with loss of cell wall integrity. Likewise, Nanjo et al (2011) showed that when soybean roots were stressed in low oxygen conditions, there was down regulation of genes related to flavonoid biosynthesis, jasmonate synthesis, and cellulose synthesis that are involved in defense response and other cellular functions. Consequently, these cellular changes may contribute to increased susceptibility of soybean seedlings to pathogen infection when flooding or low-oxygen conditions are present.

**Flooding and ethylene accumulation**

One of the most significant changes that happens during flooding conditions is the accumulation of ethylene hormone in soil and plant organs (Sasidharan and Voesenek 2015). Up-regulation of genes involved in ethylene biosynthesis was observed in soybean roots in response to flooding (Valliyodan et al. 2014). Singh and coworkers (2004) described the correlation between anaerobic soil conditions and resistance to rice blast disease; rice plant mediation of ethylene production in response to anaerobic conditions enhances rice resistance against *Magnaporthe grisea* infection.

**Ethylene biosynthesis and signaling**

Ethylene is a gaseous hormone that regulates diverse plant developmental and physiological processes, such as seed germination, flower and leaf senescence, fruit ripening, and response to biotic and abiotic stresses (Abeles et al. 1992; Broekaert et al. 2006). In plants, the first step of ethylene synthesis is the conversion of the amino acid methionine to S-adenosyle-methionine (Ado/Met) by the enzyme S-adenosyle-methionine synthase. Ado/Met is converted by the enzyme 1-aminocyclopropane-1-carboxylic acid synthase (ACS) to 5'-methylthioadenosine, and then the later is converted back to methionine through the Yange-cycle and to the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), which is the considered the rate-limiting step in ethylene biosynthesis. Finally, ACC is oxidized by ACC oxidase to ethylene, cyanide, and carbon dioxide (Yang and Hoffman 1984; Wang et al. 2002).

After its biosynthesis, ethylene binds to a family of membrane-localized receptors, which were first identified in Arabidopsis: ethylene response 1(ETR1), (ETR2), ethylene response sensor (ERS1), (ERS2), and $EIN_4$ (Bleecker 1999; Wang et al. 2002). These receptors are involved in regulation of the ethylene-signaling pathway. In the absence of ethylene, receptor

ETR1 activates the constitutive triple response 1CTR1), which suppresses ethylene response by direct phosphorylation of EIN2, which keeps EIN2 inactive (Shakeel et al. 2013). Binding of ethylene to the receptors inactivates CTR1, which in turn restores EIN2 activation. Once EIN2 is activated, its C-terminal domain is released and migrated to the nucleus to activate the transcription factors EIN3 and EIL1, either directly or indirectly. The transcription factors EIN3 and EIL1 are then accumulated in the nucleus and bind through EIN3-binding sites to the promoters of target genes such as ethylene response factor (*ERF1)*. Consequently, ERF1 will recognize and bind to GCC element in the promoters of ethylene secondary response genes, such as pathogenesis-related proteins (Cho and Yoo 2014).

**Modulation of plant defenses by ethylene**

In order to adapt to a wide variety of biotic and abiotic stresses, plants evolved defense mechanisms to perceive and respond to external stimuli (Bostock et al. 2014). One important mechanism used by plants in response to stress is the accumulation of phytohormones such as salicylic acid, jasmonic acid, and ethylene. These three phytohormones are key signaling molecules that can cross-talk with one another to fine tune an appropriate down-stream defense response (Pieterse et al. 2009). Generally, it has been accepted that salicylic acid contributes to resistance against biotrophic pathogens, while ethylene and jasmonic acid synergistically activate defenses against necrotrophic pathogens (Glazebrook 2005). Studies show that ethylene signaling is involved in modulation of pathogen-induced defenses such as i) reinforcement of physical barriers, ii) biosynthesis of antimicrobial secondary metabolites (phytoalexins), and iii) induction of pathogenesis-related (PR) proteins (Broekaert et al. 2006).

In response to pathogen attack or wounding, plants immediately accumulate physical barriers to restrict further pathogen invasion (Freeman 2008). A study by VanderMolen and coworkers (1983) showed that ethylene is required for xylem occlusion, which prevents further spread of the vascular pathogen *Fusarium oxysporum*. Several studies showed that ethylene treatment induced accumulation of hydroxyproline-rich proteins, which are structural components found in cell walls that contribute to cell wall strength and fortification (Brisson et al. 1994). Esquerre-Tugaye et al. (1979) showed that treating muskmelon seedlings with ethylene enriched the cell wall with hydroxyproline-rich glycoprotein and induced defense against anthracnose disease caused by *Colletrotrichum lagenarium*.

Another mechanism used by plants against pathogen attack is the induction of chemical defenses such as antimicrobial compounds and pathogenesis-related proteins (Maor and Shirasu 2005). Phytoalexins are secondary metabolites that have biological activity against a variety of pathogens (Ahuja et al. 2012). Several studies revealed the involvement of ethylene in phytoalexin production in different plant species (Fan et al. 2000; Nakazato et al. 2000; Broekaert et al. 2006). For instance, applications of ethylene and jasmonic acid were found to synergize the induction of maize phytoalexins such askauralexins and zealexins (Schmelz et al. 2011). In *Nicotiana benthamiana*, expression of genes for phytoalexin biosynthesis was induced by ethylene treatment (Shibata et al. 2010). On the other hand, Adie et al. (2007) found no evidence for camalexin regulation by ethylene in *Arabidopsis* which suggests that the role of ethylene in regulation of phytoalexins is type dependent (Broekaert et al. 2006). Pathogenesis-related (PR) proteins are encoded by plants in response to pathogen attack. They are grouped into 17 classes, according to protein structure and function (Adie et al. 2007). These proteins exert highly specific antimicrobial activity against fungal and bacterial species (van Loon and van Strien 1999).

Previous studies revealed that distinctive PR gene classes that have a GCC-box element in the promoter region are responsive to ethylene; examples are *PR-2* (β-1,3-glucanases), *PR-3* (basic-chitinases), *PR-4* (hevein-like), and *PR-12* (plant defensins, PDFs) (Van Loon et al. 2006; Adie et al. 2007).

**Discrepancy of the ethylene role in plant disease**

Several studies have shown that ethylene has a role in the development of disease resistance, because it induces the expression of phytoalexins and pathogenesis-related proteins (Ecker and Davis 1987; Broekaert et al. 2006). However, ethylene signaling may act as a positive or negative regulator of disease resistance, depending on pathogen life style and the plant species (Van Loon et al. 2006, Adie et al. 2007). For example, exogenous application of ethylene or ethephon (an ethylene-releasing substance) induces resistance against charcoal rot in *Medicago truncatula* caused by *Macrophomina phaseolina* (Gaige et al. 2010), rice blast caused by *Magnaporthe grisea* in rice (Singh et al. 2004), *Phytophthora capsici* in pepper (Nunez-Pastrana et al. 2011), and *Botrytis cinerea* in grape (Belhadj et al. 2008). Transgenic rice with inducible ethylene production showed enhanced disease resistance against the fungal pathogens *Magnaporthe oryzae* and *Rhizoctonia solani* (Helliwell et al. 2013). Moreover, plant mutants that are impaired in ethylene perception are more susceptible to disease. For instance, ethylene-insensitive tobacco plants grown in non-autoclaved potting soil failed to withstand non-pathogenic soil-borne fungi (Knoester et al. 1998). On the contrary, other studies demonstrated that ethylene may act as virulence factor and play a role in disease development (O'Donnell et al. 2003; Balaji et al. 2008). For example, ethylene-insensitive tomato mutants were more resistant

against bacterial diseases caused by *Clavibacter michiganensis* subsp. *michiganensis*, *Pseudomonas*, and *Xanthomonas* (Balaji et al. 2008; Scherm et al. 1998; Bent et al. 1992).

**The role of ethylene in soybean diseases**

The effect of ethylene on soybean disease resistance is inconsistent. Different effects have been observed, depending on plant pathosystems and environmental conditions. In some studies, response to ethylene enhances disease resistance. For instance, treatment with ACC (a precursor of ethylene biosynthesis) significantly enhanced resistance against *Phytophthora sojae* (Sugano et al. 2013).Soybean mutants with reduced sensitivity to ethylene showed more severe symptoms in response to infection by *Sclerotinia sclerotiorum*, *Septoria glycines* and *Rhizoctonia solani* compared to wild type plants (Hoffman et al. 1999; Bent et al. 2006). A recent study by Lanubile et al. (2015) showed that a soybean genotype with partial resistance expresses genes involved in ethylene biosynthesis only when challenged with a pathogenic *Fusarium oxysporum* isolate. In contrast, other studies showed that ethylene increased disease susceptibility. For example, ethylene-insensitive soybean mutants developed less severe symptoms in response to infection by *Pseudomonas syringae* pv. *glycinea* (Hoffman et al. 1999). Also, transcriptomic analyses of soybean roots and leaves showed that ethylene biosynthesis was induced in response to *Fv* infection (Radwan et al. 2011; 2013).

## Research Justification

Soybean production in the US represents 33% of the world's total production. However, yield losses due to the effects of abiotic and biotic stresses still constrain soybean production in the US (Hartman et al. 1999). Sudden death syndrome (SDS), caused by *Fusarium virguliforme*, has been

a problem to soybean growers since its first report in 1971 in Arkansas (Roy et al. 1997). In the last 20 years, SDS has been one of the top ten diseases that suppress soybean yield in the U.S, with average yield losses ranging from 3.7 to 75.7 million bushels, estimated from 28 states during the period of 1996 to 2007 (Wrather and Koenning, 2009). Flooding is the second most important abiotic cause of damage to soybean production (Bailey-Serres et al. 2012). Flooding at early vegetative stages for 3 days and 6 days resulted in up to 20% and 93% yield loss, respectively (Sullivan et al. 2001). In other pathosystems, the association of flooding increases the severity of soybean disease epidemics. For instance, diseases caused by *Phytophthora* and *Pythium* species are more severe during flooding conditions (Kirkpatrick et al. 2006b, 2006a; Dorrance et al. 2003b). Moreover, at the molecular level it has been shown that flooding and low oxygen stress have negative impacts on the expression of genes and proteins related to cellulose synthesis, cell wall lignification, defense mechanisms and energy metabolism; these negative impacts result in cellular injuries and susceptibility of soybean to pathogen infection (Nanjo et al. 2011).

It is well established that high soil moisture, due to excessive rainfall, irrigation, or poor soil drainage, increases SDS severity and the magnitude of yield loss (Melgar et al. 1994; Scherm and Yang 1996; De Farias Neto et al. 2006; Leandro et al. 2013). SDS epidemics are particularly severe in years where flooding occurs. The first report of SDS in Iowa occurred in 1993, which was a flood year (Munkvold 1995), and the widespread SDS outbreaks of 2008 and 2010 are also years with excessive flooding (Leandro et al. 2013).

Flooding stress is accompanied by change in physical and chemical soil properties (Kozlowski 1984). A key change that occurs during flooding conditions is a decrease in oxygen levels and build-up of $CO_2$ and toxic compounds, which creates an anaerobic soil environment

(Kozlowski 1984; Boru et al. 2003). Previous research has shown that lack of oxygen supply due to flooding or waterlogging is accompanied by soybean root injuries and reduction in soybean growth and development (Linkemer et al. 1998; Henshaw et al. 2007). Also, low oxygen due to flooding stress causes down-regulation of genes involved in biosynthesis of key defense-related genes (Nanjo et al. 2011). Moreover, several studies have associated flooding stress with the increase of soybean disease epidemics (Kirkpatrick et al. 2006b, 2006a; Dorrance et al. 2003a).

Despite the fact that flooding and the accompanying anaerobic conditions create a conducive environment for many soilborne pathogens, it is unclear if the increased disease severity under such conditions is caused by the physiological stresses that are imposed on soybean during flooded or anaerobic conditions, or by stimulation of pathogen virulence, or by both factors. Thus, elucidating the effect of flooding duration, and the influence of changes in soil oxygen and carbon dioxide gas concentrations that are typical of flooding conditions, are necessary for better understanding of the soybean-SDS pathosystem. In this study, we hypothesized that flooding and the associated reduction in soil oxygen level would increase SDS severity compared to no flooding and normal oxygen conditions.

Another change that happens during flooding conditions is the accumulation of ethylene hormone in soil and plant organs (Sasidharan and Voesenek 2015). Up-regulation of genes involved in ethylene biosynthesis was observed in soybean roots in response to flooding (Valliyodan et al. 2014). Studies have shown that ethylene has a role in the development of disease resistance, as it induces the expression of phytoalexins and *PR* genes (Ecker and Davis 1987; Broekaert et al. 2006). However, ethylene signaling may act as a positive or negative regulator of disease resistance, depending on pathogen life style and plant species (Van Loon et al. 2006; Adie et al. 2007). For example, exogenous application of ethylene or ethephon (ethylene

releasing substance) induces resistance against different pathogens such as *Macrophomina phaseolina* in *Medicago truncatula* (Gaige et al. 2010), *Magnaporthe oryzae* in rice (Singh et al. 2004), *Phytophthora capsici* in Habanero pepper (Nunez-Pastrana et al. 2011), and *Botrytis cinerea* in grape (Belhadj et al. 2008). In contrast, other studies have shown that ethylene may act as a virulence factor and play a role in disease development (O'Donnell et al. 2003; Balaji et al. 2008). For instance, ethylene-insensitive soybean mutants developed less severe symptoms in response to *Pseudomonas syringae* pv. *glycinea* and *Phytophthora sojae* (Hoffman et al. 1999).

In the soybean-*F. virguliforme* pathosystem, a trascriptome analysis revealed an up-regulation of genes that are responsible for ethylene biosynthesis in soybean roots in response to Fv infection (Radwan et al. 2011, 2013). To date, no work has been done to understand the effect of ethylene on soybean response to SDS, and it is unclear if manipulation of ethylene biosynthesis affects SDS resistance positively or negatively. Therefore, in this study, we investigated the role of ethylene in the soybean-Fv interaction by applying ethylene-inducing and ethylene-suppressing products to manipulate endogenous soybean ethylene levels.

## Research Objectives

1) To determine the effect of flooding duration on SDS disease severity
2) To investigate the effect of different oxygen and carbon dioxide concentrations on *F. virguliforme* gene expression and soybean defense-related genes in infected plants
3) To understand the role of ethylene in soybean SDS disease development
4) To develop ethylene-based management tools for SDS

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# CHAPTER 2

## SOYBEAN SUDDEN DEATH SYNDROME CAUSED BY *FUSARIUM VIRGULIFORME* IS IMPARED BY PROLONGED FLOODING AND ANAEROBIC CONDITIONS

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### Abstract

High soil moisture usually favors soybean sudden death syndrome (SDS), caused by *Fusarium virguliforme* (*Fv*), but the effects of flooding duration and flood-associated anaerobic conditions on the soybean-*Fv* interaction are not known. Greenhouse studies were conducted using susceptible and resistant cultivars exposed to the following treatments: 3, 5, or 7 days of continuous flooding, repeated short-term flooding of 8 h/week for 3 weeks, and a no-flood check treatment. At 7, 14, and 21 days after flooding (DAF), seedlings in the no-flood, 3-day, and repeated short-term treatments showed the highest root rot and foliar symptom severity, whereas

seedlings in the 7-day treatment showed the lowest severity. *Fv* inoculum density in soil was lowest in the 7-day flooding treatment. In a hydroponic system, the steady transcript levels of soybean defense genes and *Fv* candidate virulence genes were measured in response to different oxygen levels using qPCR. *Fv*-infected roots exposed to 12 h of anaerobic conditions showed down regulation of the defense-related soybean genes *Laccase*, *PR3*, *PR10*, *PAL*, and *CHS*; the *Fv* virulence gene pectate lyase (*PL*); and an *Fv* homologue of pisatin demethylase (*PDA*). Our study suggests that short term flooding tends to increase SDS, while prolonged flooding negatively impacts SDS due to reduction of *Fv* density in soil. Moreover, anaerobic conditions down-regulate both soybean defense genes and *Fv* candidate virulence genes.

## Introduction

Soybean [*Glycine max* (L.) Merrill] sudden death syndrome (SDS), caused by the soilborne fungus *Fusarium virguliforme* (*Fv*), is a devastating disease in North and South America (Covert et al. 2007). In the last two decades, SDS has been ranked among the top ten soybean diseases in the U.S. with average economic losses estimated at 190 million dollars annually between 1999 and 2004 due to yield reduction (Wrather and Koenning 2006; Leandro et al. 2012). Under cool, wet soil conditions, the fungus causes root rot and reduces root biomass. Later in the season, usually during soybean reproductive stages, the fungus secrets toxins that cause foliar interveinal chlorosis, necrosis, premature defoliation, and pod abortion (Jin et al. 1996; Roy 1997). SDS epidemics are highly dependent on environmental conditions, such as temperature and high soil moisture, both at vegetative and reproductive growth stages (Scherm et al. 1998; Gongora-Canul and Leandro 2011, Leandro et al. 2013; Scherm and Yang 1996). Thus, understanding the effect of temperature and high soil moisture on the *Fv*-soybean interaction is

important for improving SDS disease management strategies, which currently rely primarily on the use of host resistance (Hartman et al. 2015).

Flooding stress due to excess rainfall and/or poorly drained soil is considered the second most damaging abiotic stress to crop production after drought (Bailey-Serres and Colmer 2014; Setter and Waters 2003; Mittler and Blumwald 2010). In the U.S., waterlogging adversely affects about 16% of soils (Boyer 1982). Soybean is highly sensitive to flooding stress (Wuebker et al. 2001); pre-emergence flooding for as brief as 24 h can reduce emergence by 50%, and flooding at early vegetative or reproductive stages reduces soybean yield by up to 43%, and 56%, respectively (Sullivan et al. 2001; Scott et al. 1989). In addition, flooding and saturated soils can predispose soybeans to pathogen infection (Bostock et al. 2014; Kirkpatrick et al. 2006b, 2006a; Dorrance et al. 2003a). For example, root rot and damping-off caused by *Pythium* spp., *Phytophthora* spp., *Rizoctonia solani*, and *Fusarium* spp. are more severe in flooded or high soil moisture fields than in non-flooded fields (Dorrance et al. 2003b; Kirkpatrick et al. 2006b, 2006a). Similarly, wet soils, irrigated fields, and years with above average rainfall or flooding events favor SDS (De Farias Neto et al. 2006; Melgar et al. 1994; Scherm and Yang 1996; Leandro et al. 2013), and major outbreaks in Iowa have coincided with years of extreme flooding: 1993, 2008, and 2010 (Leandro et al. 2013). However, there is no information about how and why excessive soil moisture is associated with severe SDS, nor how soybeans respond to these conditions.

Flooding triggers a decrease in oxygen levels and a build-up of $CO_2$ and toxic compounds in the root zone (Boru et al. 2003; Kozlowski 1984). In soybean, lack of oxygen supply is accompanied by inhibition of respiration and mineral uptake, reduction in leaf photosynthesis, suppression of root nodulation, root injuries, and reduced growth and development (Linkemer et

al. 1998; Henshaw et al. 2007). Flooding or low-oxygen stress causes down-regulation of genes involved in biosynthesis of phenylpropanoids, lignin, and flavonoids, which play important roles in defense against plant pathogens (Nanjo et al. 2011). Suppression of root growth and lateral root formation were also observed in flooded soybean due to down regulation of genes involved in cellulose synthesis (Nanjo et al. 2011).

To protect themselves against biotic stress, plants possess a battery of defense mechanisms, including physical and chemical barriers to prevent pathogen infection and further spread (Bostock et al. 2014). For example, studies on the soybean-*Fv* interaction (Radwan et al. 2011; Yuan et al. 2008; Iqbal et al. 2009) reported that infected roots had increased transcript levels of defense-related genes, including: i) pathogenesis-related proteins such as *PR-1*, *PR-5*, and *PR-10*, ii) genes associated with the phenylpropanoid pathway such as phenylalanine ammonia lyase (*PAL*) and chalcone synthase (*CHS*), and iii) genes involved in cell wall lignification, such as laccase. On the other side, pathogens produce pathogenicity-related proteins or enzymes and virulence factors to invade their hosts and cause disease (Maor and Shirasu 2005). For instance, *Fv* secretes a low molecular weight 13.5-kDa acidic protein, named FvTox1, that causes the development of SDS leaf symptoms on soybeans (Pudake et al. 2013). Similarly, the pea pathogens *F. oxysporum* f. sp. *pisi* and *Nectria haematococca* (teleomorph of *F. solani*) produce pisatin demethylase (*PDA*) to detoxify pisatin, a phytoalexin in peas (Coleman et al. 2011). In *Alternaria brassicicola* the causal agent of black spot disease of *Brassica* species, deletion of the pectate lyase (*PL*) gene reduced virulence by approximately 30% compared to the wild-type strain (Cho et al. 2015). The role of *PDA* and *PL* on *Fv* virulence is unknown. Research is also needed to understand how these defense mechanisms are affected during *Fv* infection in combination with the abiotic stress resulting from flooding and low oxygen.

It is unclear if increased SDS disease severity under high soil moisture is caused by physiological stress on soybean, or by stimulation of *Fv* activity, or both factors. In this study, we hypothesized that flooding and the associated reduction in soil oxygen level would increase SDS severity compared to no flooding and normal oxygen conditions. The objectives of this study were to assess the effects of 1) flooding duration on SDS disease development in greenhouse plants, and 2) low oxygen levels in a hydroponic system on the soybean-*Fv* interaction by measuring the expression of the soybean defense genes and *Fv* candidate virulence genes.

## Materials and Methods

### Inoculum preparation

*Fv* isolate Mont-1 was used as the inoculum source in all trials. For spore suspension preparation, cultures were grown on potato dextrose agar media (PDA) for 28 days, at room temperature, in darkness. Plates were flooded with 20 ml of sterile distilled water, conidia were dislodged with a rubber policeman, and suspensions were filtered through a double layer of sterile cheesecloth. The conidia were quantified using a hemocytometer and adjusted to $10^6$ conidial/ml using sterile distilled water. Inoculum was prepared following the procedure described by Munkvold and O'Mara (2002). A mixture of sand (1900 ml), corn meal (380 ml), and distilled water (110 ml) was autoclaved in 20 cm X 30 cm bags (Fisher scientific, Pittsburg, PA), for 1 hour at 121°C, on two consecutive days. Each bag was then inoculated with 2 ml of the spore suspension, or with 2 ml of sterile distilled water for controls. The bags were incubated in the dark, at room temperature, for 6 days, with daily mixing by hand, to keep uniform distribution of the fungus.

**Effect of flooding on SDS**

Soybean cultivars Williams 82 (susceptible to SDS) and MN1606 (resistant to SDS) were used in all greenhouse experiments. Before planting, the prepared *Fv* inoculum was mixed, at a ratio of 1:10 (v/v), with a 2:1 mixture of pasteurized sand:soil. Non-infested soil was used for controls. Styrofoam cups (236 ml) were filled with the infested mixture, and four seeds were sown per cup, 2 cm below the soil surface. Plants were grown in the greenhouse at 24°C and a 16-h photoperiod, with daily watering to avoid dehydration. At the unifoliate stage (Fehr et al. 1971), seedlings were thinned to two plants per cup, then exposed to different flooding periods by submerging the cups in plastic basins (88.1 cm L x 41.9 cm W x 15.2 cm H) and maintaining the water level 2 cm above the soil surface during the flooding periods. The unifoliate stage was selected because soybean are particularly susceptible to flooding stress at this stage (Sullivan et al. 2001; Scott et al. 1989), and to expose the plants to flood stress before SDS foliar symptoms typically appear in greenhouse conditions (Gongora-Canul and Leandro 2011). The flooding treatments were as follows: continuous flooding for 0 days (no-flood control), 3, 5, and 7 days, or a repeated short-term flooding of 8h/week for three weeks. The repeated short-term treatment was applied by exposing the plants to 8 h of continuous flooding in the first day of each week, and repeating this process for two more weeks (Sah et al. 2006). After each flooding period, the cups were moved to the bench and allowed to drain, and then regular watering resumed. For the no-flood control treatment, plants were maintained on the bench and watered as needed to avoid dryness.

A 2 × 5 × 2 factorial experiment encompassed two cultivars (Williams 82, and MN1606), five flooding treatments (no flood, RS, 3, 5, and 7 days), and two Fv inoculation levels (inoculated and non-inoculated), in a completely randomized design. There were 10 replicate cups per treatment combination for each of three assessment times, and the experiment was conducted three times. Twenty plants (two per each of ten cups) were destructively sampled and used for root rot and foliar disease ratings 7, 14, and 21 DAF (days after start of flooding). Root rot and foliar symptom severity were rated visually as the percentage of root area showing brown or black discoloration, and percentage of leaf area showing chlorosis and/or necrosis, respectively. Area under disease progress curve (AUDPC) was calculated for SDS foliar and root rot severity (Simko and Piepho, 2012). Root and shoot dry weight were measured on five arbitrarily selected plants (one per cup) by rinsing in tap water to remove soil particles, and drying the root and shoots separately in an oven at 70°C for 1 or 2 days.

*Fv* population density in soil was assessed in three randomly selected cups planted with Williams 82, following protocols of Rupe et al. (1997). One gram of soil was placed in 100 ml of sterile distilled water in a 250-ml flask, and 10-fold serial dilutions were prepared ranging from $10^{-1}$ to $10^{-3}$. A volume of 0.2 ml was transferred from each $10^{-3}$ dilution and spread over two plates of modified Nash and Snyder's medium (MNSM) using a rubber policeman. The $10^{-3}$ dilution was used as it showed countable number of *Fv* colonies in preliminary tests. There were a total of 6 plates (3 cups x 2 plates) per treatment. The plates were incubated at room temperature for 5 days in the dark, and the numbers of colonies obtained were used to calculate the number of colonies forming units per gram of soil (CFU/g soil).

**Effect of oxygen level on the soybean – *Fv* interaction**

To examine the effect of anaerobic (no oxygen) and hypoxic (low oxygen) conditions on the soybean-*Fv* interaction, we analyzed the expression of selected host defense related genes and *Fv* candidate virulence genes in *Fv*-inoculated soybean roots subjected to different oxygen levels, as described below. Soybean defense related genes studied were *Laccase*, *PR-3*, *PR-10*, *PAL*, and *CHS*. *Fv* virulence genes studied were *FvTox1*, *PDA*, and *PL*. The *PDA* and *PL* genes were selected because they are homologues of genes with known virulence functions in the pea pathogens *F. oxysporum* f. sp. *pisi* and *N. haematococca.* For instance, pisatin demethylase is an enzyme produced by microbial pathogens such as *F. oxysporum* to detoxify the plant phytoalexin pisatin (Coleman et al. 2011). Pectate lyase is an pectin-digesting enzyme that plays important role in pathogen invasion and colonization of host tissue by degrading pectin in plant cell walls and middle lamellae (Cho et al. 2015).

***Validation of the PDA and PL genes predicted to be involved in Fv virulence.*** An experiment was conducted to analyze the differential expression of *Fv* candidate virulence gene *PDA* and *PL* in mycelia, germinated conidia, and soybean roots. The Mont-1 isolate was maintained on Bilay agar plates [(0.1% $KH_2PO_4$ (w/v), 0.1% $KNO_3$ (w/v), 0.05% $MgSO_4$ (w/v), 0.05% KCl (w/v), 0.02% starch (w/v), 0.02% glucose (w/v), 0.02% sucrose (w/v) and 2% agar (w/v)] and sub-cultured on 1/3 PDA agar plates [0.04% potato starch, 0.2% glucose, 2% agar (w/v)]. To prepare infected plant material, soybean cultivar Williams 82 was sown in plastic containers (50.8 cm L x 26.67 cm W x 6.35 cm H) containing coarse vermiculite, and maintained under dark conditions with a temperature schedule of 16°C for 8 h, 2°C increases every h for 4 h,

27°C for 8 h, and 2°C decreases every h for 4 h. Seven-day-old seedlings were uprooted, and vermiculite was gently washed from roots with tap water. Three seedlings were placed in a 50 ml conical tube with 25 ml of a *Fv* conidial suspension ($10^6$ conidia/mL). For the non-inoculated control plants, roots were immersed in 25 ml of sterile water. Seedlings were incubated at 22°C in the dark for 1, 3, 5, and 10 days after inoculation (DAI). The experiment was set up as a 2 × 4 factorial, with two *Fv* inoculation levels ($10^6$ conidia/mL and 0 conidia/mL) and four sampling times (1, 3, 5, and 10 DAI), in a completely randomized design. For each sampling time, the roots of the three seedlings in each tube were pooled, immediately frozen in liquid nitrogen and stored in -80$^0$C until RNA preparation. The experiment was repeated two more times.

To prepare *Fv* mycelia, plugs from an *Fv* culture in solid Bilay media were transferred to liquid modified Septoria media (MSM) (Jin et al. 1996), and incubated in darkness, at room temperature, for 2 weeks. The mycelia were then harvested, liquid medium removed by vacuum filtration, and dried mycelial tissues immediately frozen in liquid nitrogen, and stored in -80°C until use for RNA preparation. To obtain germinating conidia, conidia were harvested from 1/3 strength PDA plates and germinated in 10 ml MSM for 12 h. Conidia were centrifuged for 5 min at 6000 g and supernatant was removed. Conidia were washed 2 times with distilled water to remove residual media. Washed conidia were used directly for RNA preparation. For each sampling time, there were three replicate flasks of MSM for growing mycelium, and three replicate plates of 1/3 PDA for collecting conidia.

***Hydroponic experiment.*** A hydroponic system was established under controlled environment to investigate the effect of different oxygen levels on soybean defense related genes and *Fv* candidate virulence genes. Soybean seeds of the SDS resistant and susceptible varieties were sown in 236 ml Styrofoam cups filled with sand-soil mixture infested with *Fv* as described

above. The plants were then incubated at 24°C with a photoperiod of 16 h until the unifoliate stage (10-12 days after planting) to allow infection to occur. The pre-infected seedlings were then transferred to plastic containers (5.6 liters) containing 4 liters of full-strength Hoagland's nutrient solution (MP Biomedicals, LLC, Santa Ana, CA). A perforated Styrofoam sheet supported seedlings, and the root system was suspended in the solution.

The experiment consisted of three oxygen level treatments: 1) normal air conditions (21% $O_2$), 2) hypoxic conditions (4% $O_2$ + 10% CO2 + 86% $N_2$), and 3) anaerobic conditions (0% $O_2$, 100% $N_2$). Plants were kept in the nutrient solution trays 24 h before exposure to the gas treatments to allow adaptation to the hydroponic environment. The gas treatments were injected from premixed gas cylinders (Praxair Technology, Inc., Des Moines, IA) and bubbled into the nutrient solution. For each gas treatment, there were two plastic containers, each with 12 plants (2 varieties × 2 sampling times ×3 replicate plants). Seedlings were collected at 6 and 12 h after the start of exposure to the oxygen treatments. In order to validate the hydroponic system, oxygen concentration of the nutrient solution was monitored using an oxygen meter (model MW600, Milwaukee instruments, Milwaukee, WI). In addition, the expression level of the anaerobic marker gene alcohol dehydrogenase (*ADH1*) was measured at 6 and 12 h, and the pH of the nutrient solution was adjusted to 4.5.

The experiment was conducted in three runs. Each run followed a split-plot design with gas treatment (normal, hypoxic, and anaerobic) as the main plot factor, and a 2 × 2 factorial combination of sampling times (6 and 12 h) and cultivar (resistant and susceptible) as the split plot factor. The resistant cultivar was used in all runs, while the susceptible cultivar was used in runs 2 and 3 only. To quantify change in gene expression of soybean defense related genes and *Fv* candidate virulence genes, whole roots were collected at 6 and 12 h, and immediately frozen in

liquid nitrogen and stored at -80°C until use. At each sampling time, root samples were collected by pooling 3 plants per variety, from each container, resulting in a pooled sample of 6 plants.

***RNA extraction and cDNA synthesis for quantification of gene expression using qPCR***. For RNA extraction, samples were ground to a fine powder in liquid nitrogen, and total RNA was extracted using the RNeasy mini kit (Qiagen, Germantown, MD, USA). DNA contamination was removed using RNase-free DNase I (Invitrogen) following the manufacture's procedure. RNA quantity was determined using a Nano-Drop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and integrity was verified on 1% agarose gel. For cDNA synthesis, 0.5 µg of total RNA was reverse transcribed using Superscript III and random hexamers or Oligo (dT) primers (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR was performed using Perfecta SYBR Green fast mix (Applied Biosystems, Foster City, CA, USA) and an iQ5 PCR detection system (Bio-Rad, Hercules, CA, USA). The reaction mix consisted of 10 µl master mix, 0.5 µl of reverse and forward primers (250 nM final concentration), 8 µl of diluted cDNA, and the final volume was adjusted to 20 µl with RNase DNase free water (Invitrogen). The primers used to amplify each gene are shown in Supplementary Table 1. The thermal cycling protocol was: 3 min at 95°C, 40 cycles of 10 s at 95°C, 15 s at (primer annealing temperature, and 30 s at 72°C, followed by melting curve data collection to check for non-specific amplification and primer dimers. For gene expression analysis, each treatment had 3 biological replicates with two technical replicates. Relative gene expression was calculated using the $2^{-\Delta\Delta ct}$ method (Livak et al. 2001), in which the transcript levels of the target genes in normal air treated seedlings were considered to be the basal levels during expression analysis, and β-Actin and 18s rRNA were used as internal control genes for host and pathogen, respectively.

For validation of *Fv* candidate virulence genes, the transcript levels of the target genes in mycelia were used as the basal level, and *FvTox1* as internal control gene.

**Data analysis**

*Flooding assay.* Analysis of variance was performed using SAS PROC GLIMMIX (SAS Institute, Cary, NC) to determine the main and interaction effects of treatment factors on root rot severity, foliar disease severity, *Fv* inoculum density, and plant dry weight. Fisher's Protected Least Significance Difference test was used for comparing means among flooding treatments, inoculum status, and cultivars. Data for root rot severity (%), foliar severity (%) and *Fv* density in soil (cfu $g^{-1}$) were available only for inoculated plants. Therefore, for analyses of these responses, flooding treatment and cultivar were classified as fixed effects, and replication and run were used as random effects. Foliar symptom and root rot data from the three assessment times (7, 14, and 21 DAF) were summarized by calculating area under the disease progress curve (AUDPC) (Simko and Piepho 2012). Plant dry weight data for inoculated and non-inoculated plants were analyzed to determine the effect of flooding alone and in combination with *Fv* infection. Analysis of variance was performed using PROC GLIMMIX to analyze the differential expression of *Fv* candidate virulence genes in mycelia, germinated conidia, and soybean root. Gene expression in inoculated roots was expressed as a log-fold change, relative to gene expression in mycelium grown in-vitro. Relative expression of Fv virulence genes was compared among six growth states (mycelium, germinating spores, and in soybean roots at 1, 3, 5, 7, and 10 post inoculation. For the hydroponics experiment, gene expression analyses were performed separately for each sampling time. Log-fold change in gene expression, relative to expression in seedlings in normal air conditions, was analyzed using SAS PROC MIXED. Preliminary analyses

showed that cultivar and interaction of cultivar and oxygen level did not influence gene expression; therefore, the analysis was simplified by pooling cultivars. Because experiment runs were considered to be blocks (or replications), the model used the interaction of runs and treatments as the residual error. The fixed effect of oxygen treatment was analyzed using observations for both cultivars ($n$=5; 3 runs for the resistant cultivar, plus 2 runs for the susceptible cultivar) for each sampling time.

## Results

### Effect of flooding duration on SDS

***Foliar and root rot severity.*** There were significant main effects of flooding treatment and cultivar on severity of SDS foliar and root rot symptoms at all time points tested. However, significant interactions between flooding treatments and cultivars were observed only at 7 DAF; therefore, data is shown for the main effect of flooding treatment, averaged over both cultivars. AUDPC values for foliar and root rot severity were lower in plants exposed to 5- and 7-day flooding treatments compared to all other treatments (Table 1). At 21 DAF, foliar disease (37%) and root rot severity (47%) were lowest in the 7-day flooding treatment (Fig. 1 & 2). Mean foliar disease in the no flood, repeated short-term, and 3-day flooding treatments ranged from 66 to 70% at 21 DAF, and mean root rot severity in the same 3 treatments ranged from 66 to 75% (Fig. 3). Non-inoculated plants did not show SDS foliar symptoms, and root rot severity was consistently below 10% in all non-inoculated plants (data not shown).

***Fusarium virguliforme density in soil.*** Flooding had a significant effect on *Fv* population density in soil used to grow Williams 82 plants ($P < 0.0001$). At 7, 14, and 21 DAF, *Fv* population was lower ($P < 0.0001$) in the 7-day flooding treatment than in all the other treatments (Fig. 4). No difference was observed in *Fv* population in the no-flooding treatment compared to RS, 3-day and 5-day flooding, at all assessment times, except for 21 DAF when *Fv* density was greater in non-flooded soil compared to 5-day flooding (Fig. 4).

***Plant dry weight.*** There were significant main effects of flooding ($P<0.0001$) and inoculum ($P<0.0001$) on plant root dry weight variable but no significant effect of cultivar was observed. Also, there was no significant interaction between flood, cultivar, and inoculum at 7, 14, and 21 DAF. With the exception of the 7 DAF assessment time, there was no significant interaction between flooding and cultivar; therefore, cultivars were combined to present the effect of flooding on root and shoot dry weight.

In non-inoculated plants, flooding treatment significantly affected root dry weight ($P < 0.001$). For example, at 7 and 14 DAF, plants in the no-flood and repeated short-term treatments had greater root dry weights than plants flooded for 5- and 7-days (Fig. 5). In inoculated plants, flooding for 3, 5 or 7 days reduced root dry weight compared to the no flood treatment at 7 DAF ($P < 0.001$), but not at 14 or 21 DAF. Inoculation with *Fv* reduced root dry weight in both non-flooded and flooded plants ($P < 0.001$). For example, at 21 DAF, inoculated plants in the no-flood treatment showed up to 76% reduction in root dry weight relative to non-flooded, non-inoculated plants without (Fig. 5). Inoculated plants exposed to flooding also had lower root dry weights ($P<0.001$) compared to non-inoculated plants subjected to flooding, with the highest reduction in shoot weight (84%) observed in the repeated short term flooding treatment at 21 DAF.

A reduction in root dry weight was also observed in response to inoculation at 7 and 14 DAF, but was less pronounced.

Shoot dry weight was influenced by flooding treatment and *Fv* inoculation, but not cultivar. Therefore, cultivar data were combined to examine the effect of flooding treatment on shoot dry weight. Flooding treatment affected shoot dry weights of non-inoculated plants at all assessment times ($P< 0.001$), but did not affect shoot weights of inoculated plants. Plants not exposed to flooding had higher shoot dry weights compared to 5- and 7-day flooding durations at all assessment times (data not shown). Shoot dry weight was lower ($P < 0.001$) in *Fv*-inoculated plants compared to non-inoculated plants, for all flood treatments and both soybean cultivars, at 21 DAF (data not shown).

**Effect of oxygen level on the soybean – *Fv* interaction**

***Expression of Fv PDA and PL candidate virulence gene homologues*.** At all time points examined, both *Fv* PDA and PL genes showed significant up-regulation in soybean roots compared to expression in mycelia and germinated conidia ($P < 0.0001$; Fig. 6), suggesting that both genes might be candidate virulence factors for *Fv*. The two genes showed different expression patterns over time. For example, *PDA* had the highest accumulation at 1 DPI, with expression declining at later time points, whereas the expression of the *PL* gene was highest at 10 DPI.

***Effect of oxygen level on soybean defense related genes.*** At 6 and 12 h after initial exposure to oxygen treatments, there was up-regulation ($P < 0.01$) of the *ADH1* anaerobic marker gene under hypoxic and anaerobic conditions compared to normal air, indicating that the

hydroponic system was successfully controlled. All defense-related genes tested were down-regulated in roots exposed to anaerobic conditions for 6 h compared to normal air or hypoxic conditions ($P < 0.01$), except for *PR10* where no difference was observed between treatments (Fig. 7). At 12 h, the same pattern was observed in the *Laccase*, *PAL,* and *PR3* genes ($P < 0.01$), whereas no changes were observed for the *CHS,* and *PR10* genes. For all genes tested, similar expression was observed under hypoxic and normal air conditions at both time points.

***Effect of oxygen level on F. virguliforme genes.*** At 6 h, down-regulation of the *PDA* and *FvTox1* genes were observed in roots exposed to anaerobic or hypoxic conditions, respectively, compared to normal air ($P < 0.01$), whereas the *PL* gene showed no change. At 12 h, all *Fv* genes, except *FvTox1,* showed down-regulation in roots exposed to anaerobic conditions compared to normal air conditions ($P < 0.01$). The expression of most *Fv* candidate virulence genes did not differ between normal and hypoxic treatments at both time points. However, *FvTox1* was down-regulated at 6 h, and the *PL* gene was down-regulated at 12 h (Fig. 8).

## Discussion

To the best of our knowledge, this is the first report of the effect of anaerobic and hypoxic conditions on the soybean-*Fv* interaction. This study showed that flooding influences SDS disease severity and *Fv* population density in soil under greenhouse conditions, and that the overall effect on SDS depends on duration of the flooding period. The short-term flooding periods of 3-day and repeated short-term generally predisposed soybean seedlings to SDS, whereas continuous flooding for 5- or 7-days resulted in lower SDS severity. Additionally, this study has shown that low oxygen levels representative of those that might occur during flooding conditions (4% and

0% oxygen) generally decreased the expression of soybean defense related genes and *Fv* candidate virulence genes.

Our findings on short-duration flooding support previous studies demonstrating that high soil moisture favors SDS epidemics (Scherm and Yang, 1996; Melgar et al. 1994; Scherm et al. 1998). In the resistant cultivar MN1606, our study showed that 3-days of continuous flooding was more conducive to SDS root rot and foliar symptoms than no flooding. This provides experimental support for an observational study (Leandro et al. 2013) suggesting that years with flooding events tended to coincide with the most severe SDS epidemics. However, longer-term flooding of 5- or 7-days resulted in less severe SDS compared to the shorter flooding treatments. This might explain an observation by Rupe et al. (1995) that flooding stress during soybean vegetative growth stages could delay or reduce SDS under field conditions. Flooding can also reduce incidence and severity in several other pathosystems. For instance, severity of *Verticillium* wilt of Chile pepper (*Capsicum annuum* L.) was less severe under periodic flooding compared to no-flood conditions (Sanogo et al. 2008). Similarly, Singh et al. (2004) showed that rice cultivars grown in flooded conditions were less susceptible rice to leaf blast caused by *Pyricularia grisea* compared to rice grown upland, where no flooding is used.

Our study suggests that one of the mechanisms underlying the reduction of SDS disease development in long-term flooding might be a reduction in *Fv* density in soil. A reduction in *Fv* CFU/g soil was observed after 5- and 7-days of flooding compared to the shorter duration flooding and no-flood treatments. Previous studies showed that reduction in population density of other *Fusarium* spp. in flooded soil might be attributed to the effect of anaerobic conditions (Unger et al. 2009; Bonanomi et al. 2010). For example, Ioannou et al. (1977) reported that 40 days of continuous flooding reduced microsclerotia production of *Verticillium dahliae*, and Niem

et al. (2013) showed that 18 weeks of soil flooding at 20°C reduced viability of *Sclerotinia sclerotiorum* sclerotia.

It has been previously reported that flooding at early vegetative stages reduces root and shoot dry weights of soybean (Linkemer et al. 1998). Similarly, in this study, flooding reduced root and shoot dry weights in non-infested plants exposed to 5- and 7-days of flooding compared to no-flooding, in both cultivars. In plants infected by *Fv*, flooding generally did not have an effect on plant dry weight. However, for each flooding treatment, infection by *Fv* resulted in an additional reduction in plant dry weight compared to non-inoculated, no flooding plants, suggesting an additive effect of both stresses in reducing soybean root and shoot dry weight. This is consistent with a study by Kirkpatrick et al. (2006a) showing that flooding caused an additive effect in reducing soybean dry weight when plants were infected by *Pythium ultimum*.

At the molecular level, plants subjected to flooding or low oxygen stresses have been shown to exhibit down regulation of defense responses, cell wall biosynthesis, and lignification (Drew and Lynch 1980; Nanjo et al. 2011). For example, in soybean, down-regulation of genes related to biosynthesis of phenylpropanoids, lignin, and flavonoids were observed under flooding or low oxygen stresses (Nanjo et al. 2011). Consistent with this, our study showed that anaerobic conditions down-regulated key soybean defense response genes, such as *Laccase*, *PAL*, *CHS* and pathogenesis related proteins, compared to normal oxygen levels, in inoculated plants. *Laccase* enzymes play an important role in defense response against fungal infection, including detoxification of fungal toxins and increase of cell wall lignification (Lozovaya et al. 2006). *Laccase* gene transcription abundance in *Fv*-inoculated roots might play a role in partial resistance against SDS inoculated soybean (Iqbal et al. 2009). Phenylalanine ammonia lyase (*PAL*) and chalcone synthase (*CHS*) are key enzymes in the phenylpropanoid pathway that play a

crucial role in plant defense against pathogen infection (Yuan et al. 2008). In the soybean-*Fv* interaction, accumulation of *PAL* and *CHS* in roots of resistant soybeans has been shown in response to early infection by *Fv* (Iqbal et al. 2005). Pathogenesis-related proteins play important roles in defense response against biotic and abiotic stresses (Xu et al. 2014). As a sign of host defense induction, Radwan et al. (2011) reported accumulation of 31 PR related genes in roots of *Fv*-infected soybeans. Results from our gene expression analysis suggest that down-regulation of some soybean defense related genes under low oxygen stress might be involved in the increased susceptibility of soybeans to *Fv* infection under wet soil conditions (De Farias Neto et al. 2006; Scherm and Yang 1996).

From the pathogen side, our study suggests that low-oxygen stress can affect the expression of *Fv* candidate virulence genes, but expression level is dependent on the oxygen concentration and duration of exposure. For instance, two genes tested were down-regulated in *Fv*-infected soybean seedlings exposed to 12 h of anaerobic conditions, whereas no changes were observed between hypoxic and normal conditions except for the *PL* gene. *FvTox1* is an *Fv* gene involved in causing SDS leaf symptoms (Pudake et al. 2013). Pisatin demethylase is an enzyme produced by microbial pathogens such as *F. oxysporum* to detoxify the plant phytoalexin pisatin (Coleman et al. 2011). Pecate lyase is an pectin-digesting enzyme that plays an important role in pathogen invasion and colonization of host tissue by degrading pectin in plant cell walls and middle lamellae (Cho et al. 2015).

A previous report showed that the *PDA* gene was involved in pathogenicity of the pea pathogen *N. haematococca* (Han et al. 2001) which is the closest relative of *Fv* in peas (O'Donnell 2000); however, there was no previous evidence that this gene is important in *Fv*. In the present work,

we have confirmed that the *PDA* gene is rapidly induced in infected soybean roots compared to germinating conidia and mycelium, suggesting that it plays a role in *Fv* virulence.

The mechanisms underlying differential regulation of *Fv* candidate virulence genes under low oxygen conditions are not known. Previous studies have shown that some pathogens cannot survive anaerobic stress, while other pathogens can adapt to hypoxic conditions and colonize their hosts. For instance, *F. oxysporum, Verticillium dahlia,* and *S. sclerotiorum* were unable to persist and cause disease following 4 to 6 weeks of anaerobic soil disinfestation (Lamers et al. 2010). It is possible that the down-regulation of the *PL* and *PDA* genes observed in our study was caused by inability of *Fv* to survive in anaerobic conditions, as also indicated by a reduction in *Fv* density in response to 7-days of flooding. A more comprehensive study is needed to investigate the effect of low oxygen conditions on more host defense and *Fv candidate virulence* genes and on disease development.

The results for the repeated short-term and 3-day continuous flooding treatments support our hypothesis that flooding increases SDS severity, but the opposite effect was observed with longer flooding periods. This pattern suggests that the overall effect of flooding on SDS depends on the duration of flooding and on the balance between the direct impacts of flooding on the pathogen and the host. For example, under short-duration flooding, soybeans were already stressed, as indicated by a reduction in dry weight in non-inoculated plants, but the *Fv* population was apparently not yet affected. The observed increase in SDS severity maybe therefore has been due to greater predisposition of the stressed plants to high *Fv* levels. Under longer duration flooding, plants were stressed, but *Fv* density and enzyme activity in soil were then also decreased. This resulted in reduced infection of newly formed roots over time, leading to reduced root rot severity (expressed as a percentage of total root area), and reduced foliar symptoms. Furthermore, we

observed that anaerobic and hypoxic conditions, representative of those that can occur during flooding stress, adversely affected key host defense response genes, which might explain increased SDS severity in soybean seedlings exposed to 3-days of continuous flooding compared to no flooding.

A limitation of our study is that the effects of flooding and oxygen levels on SDS were tested only on seedlings and under controlled environment conditions. Future work should validate the findings reported here by exposing plants to different flooding durations under field conditions, and also at different growth stages. If flooding is shown to predispose soybean to SDS in field conditions, future research could investigate if flood tolerant soybean genotypes show enhanced resistance to SDS. Finally, further investigation of the effects of flooding on other factors, such as antagonistic microorganisms and soil physical-chemical properties, are also needed to better understand how flooding affects *Fv* survival and pathogenicity.

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**Table 1.** Effect of flooding treatment on SDS development expressed by the area under disease progress curve (AUDPC) of foliar disease severity and root rot in two soybean cultivars [x]

| | AUDPC [y] | |
|---|---|---|
| Assessment [w] | Foliar | Root rot |
| Treatments [z] | | |
| No flood | 735.42 a | 752.11 a |
| RS | 804.99 a | 823.91 a |
| 3 | 801.16 a | 849.73 a |
| 5 | 509.78 b | 599.07 b |
| 7 | 424.45 b | 530.48 b |

[x] The data analysis showed no significant interaction between flooding treatment and cultivar, therefore, the resistant cultivar MN1606, and susceptible cultivar Williams 82 were combined for analysis.

[y] Data represents the mean area under disease progress curve (AUDPC) of SDS foliar disease and root rot severity based on three weekly visual disease ratings at 7, 14, and 21 days after flooding in both cultivar. Numbers followed by different letters within the same column are significantly different at $P<0.05$ according to Fisher's least significant difference test.

[w] Assessments: Root rot and foliar symptom severity were rated visually as the percentage of root area showing brown or black discoloration, and percentage of leaf area showing chlorosis and/or necrosis, respectively.

[z] Treatments: No flood= control, RS= repeated short term, 3= three days of continuous flood, 5= five days of continuous flood, and 7= seven days of continuous flood.

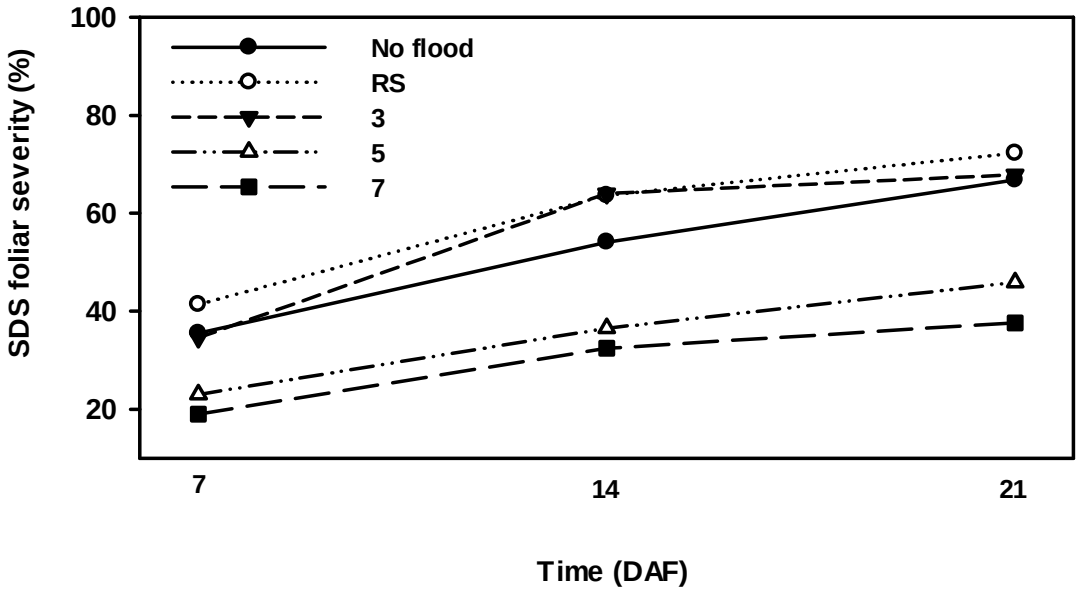


**Fig. 1.** Severity of foliar symptoms of soybean sudden death syndrome in soybean seedlings exposed to no flooding (NF), repeated short-term flooding (RS), and 3, 5, and 7 days of continuous flooding, and assessed 7, 14, and 21 days after start of flooding. Each point represents the mean of 60 replicates (10 cups x 3 runs x 2 cultivars).

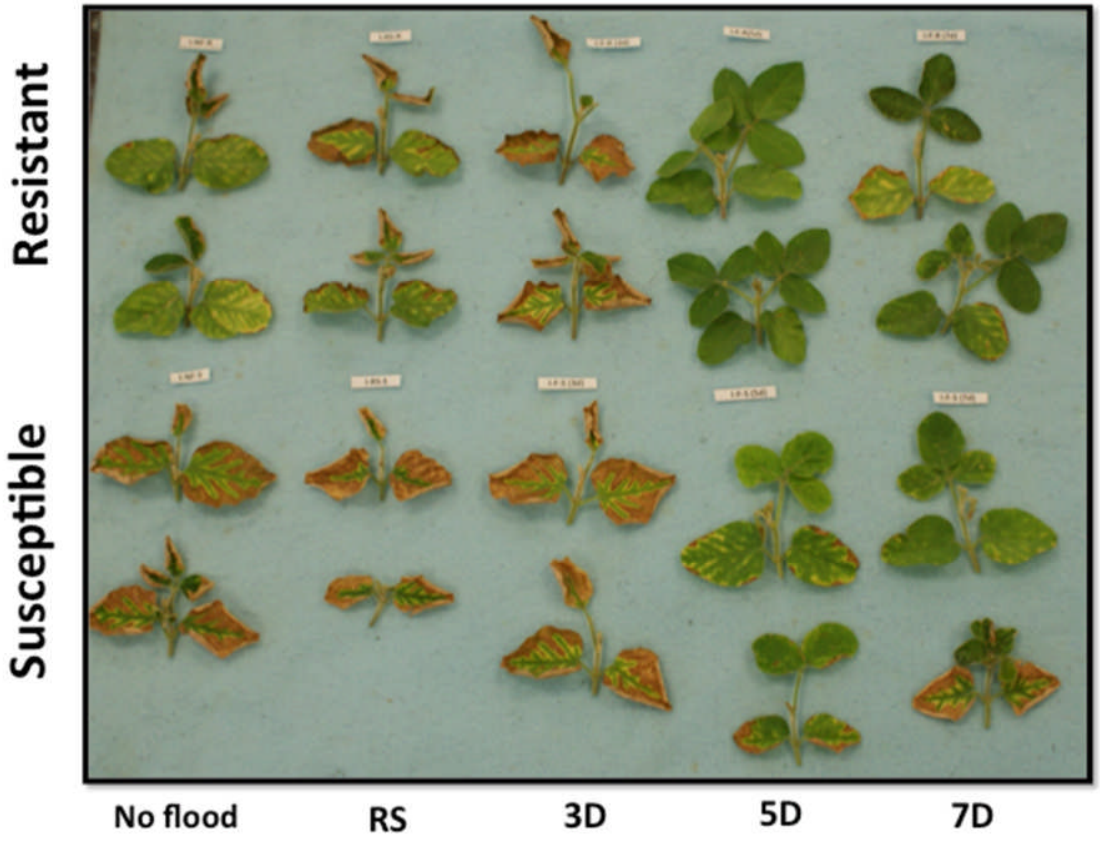


**Fig. 2.** Soybean cultivars MN1606 (SDS resistant), and Williams 82 (SDS susceptible) showing SDS foliar symptoms 21 days after exposing to no-flood (control), repeated short-term (RS), 3-day, 5-day, and 7-day flooding regimes.

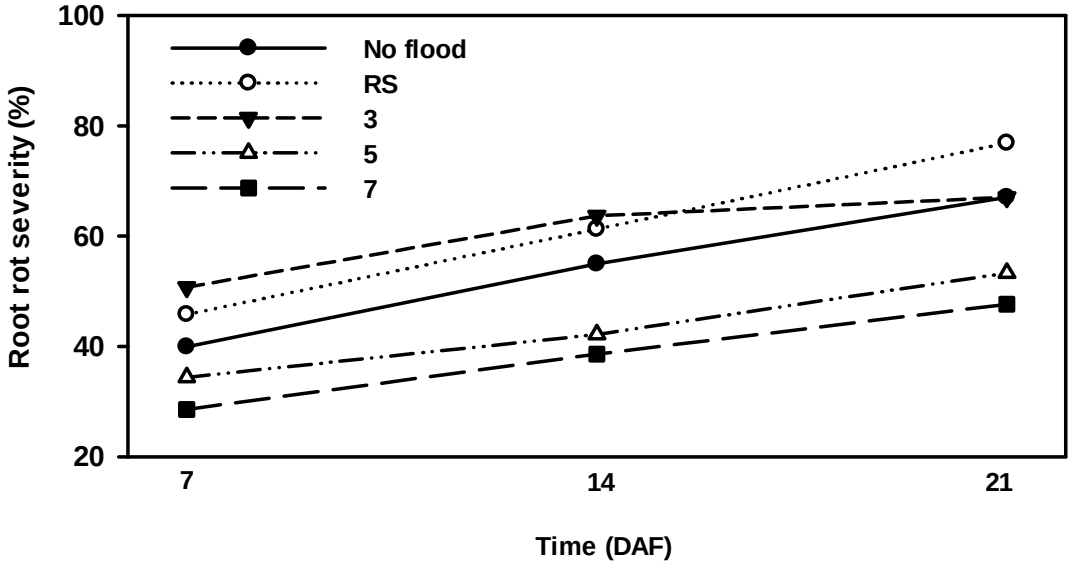


**Fig. 3.** Severity of root rot symptoms of soybean sudden death syndrome in soybean seedlings exposed to no flooding (NF), repeated short-term flooding (RS), and 3, 5, and 7 days of continuous flooding, and assessed 7, 14, and 21 days after start of flooding. Each point represents the mean of 60 replicates (10 cups x 3 runs x 2 cultivars).

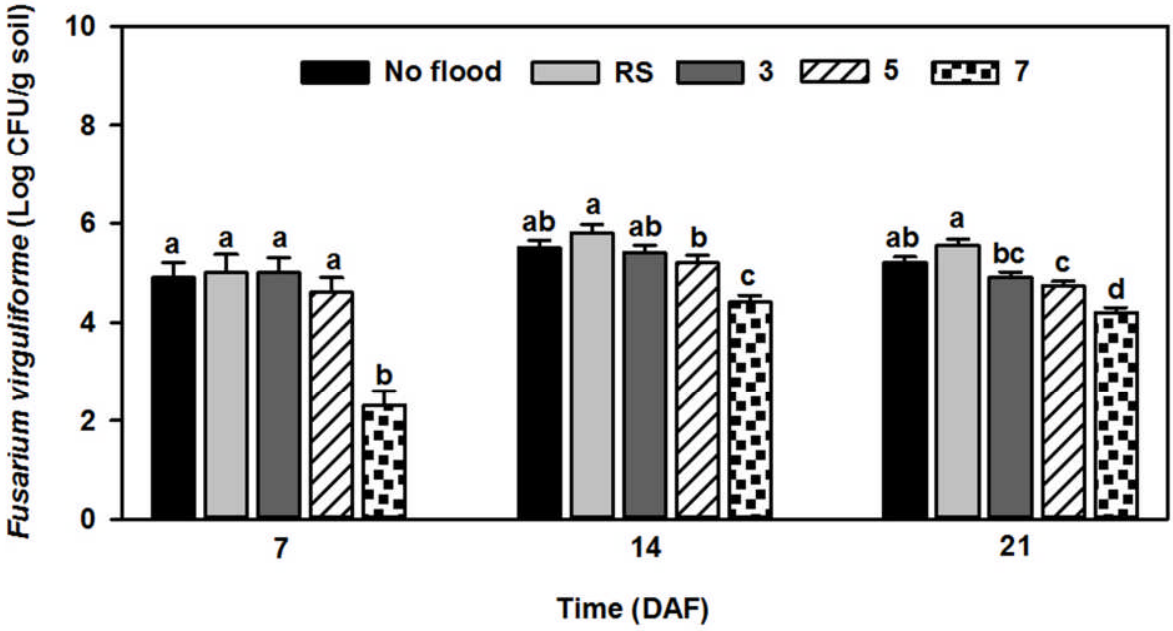


**Fig. 4.** Population density of *Fusarium virguliforme* in soil from the Williams 82 cultivar exposed to no flooding (NF), repeated short-term flooding (RS), and 3, 5, and 7 days of continuous flooding, and assessed 7, 14, and 21 days after start of flooding, Error bar represents the standard error of the mean (n=9). Means with different letters are significantly different ($P<0.05$).

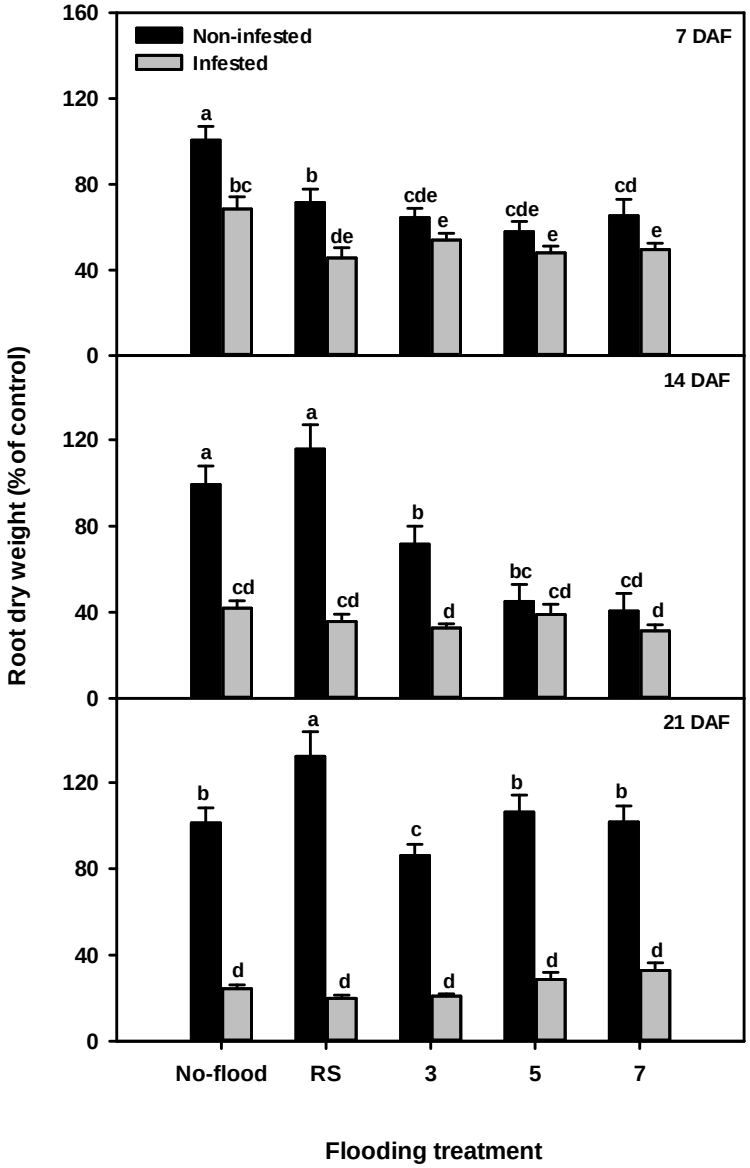


**Fig. 5.** Effect of flooding on root dry weight of soybean cultivars, inoculated or non-inoculated with *Fusarium virguliforme*, at 7, 14, and 21 days after start of flooding (DAF). Columns are the percent mean of root dry weight relative to the non-inoculated, no flooding control. Each bar represents the mean of 30 replicates (5 cups x 3 runs x 2 cultivars) and the error bar represents the standard error of the mean (n=30). Columns with different letters are significantly different ($P<0.05$).

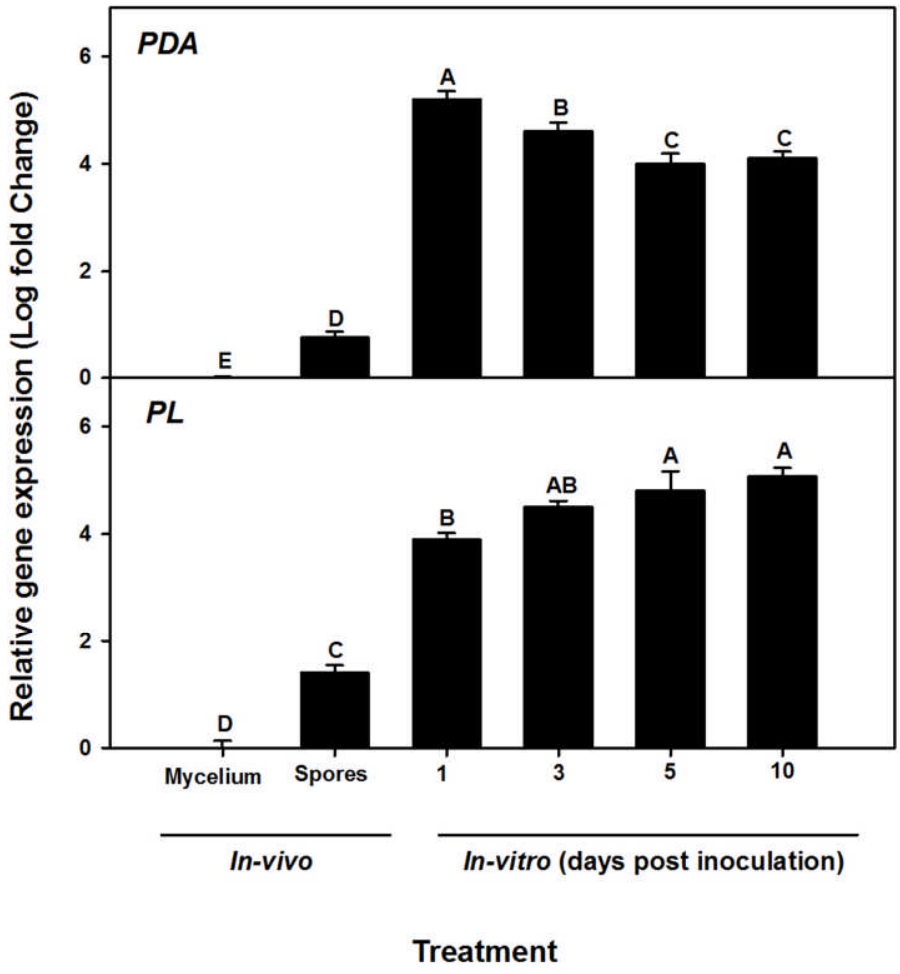


**Fig. 6.** Expression of two candidate *Fusarium virguliforme* virulence genes pectate lyase (PL), and pisatin demethylase like (PDA), in mycelia, germinated spores, and Fv infected soybean roots at 1, 3, 5, and 10 days post inoculation. FvTox1 was used as an internal control gene. Each bar represents the mean log-fold change of gene expression for three experimental runs relative to the Fv mycelia. Each treatment has three biological replicates and two technical replicates. Error bar indicates standard error of the mean (n=3). Columns with different letters are significantly different ($P<0.0001$).

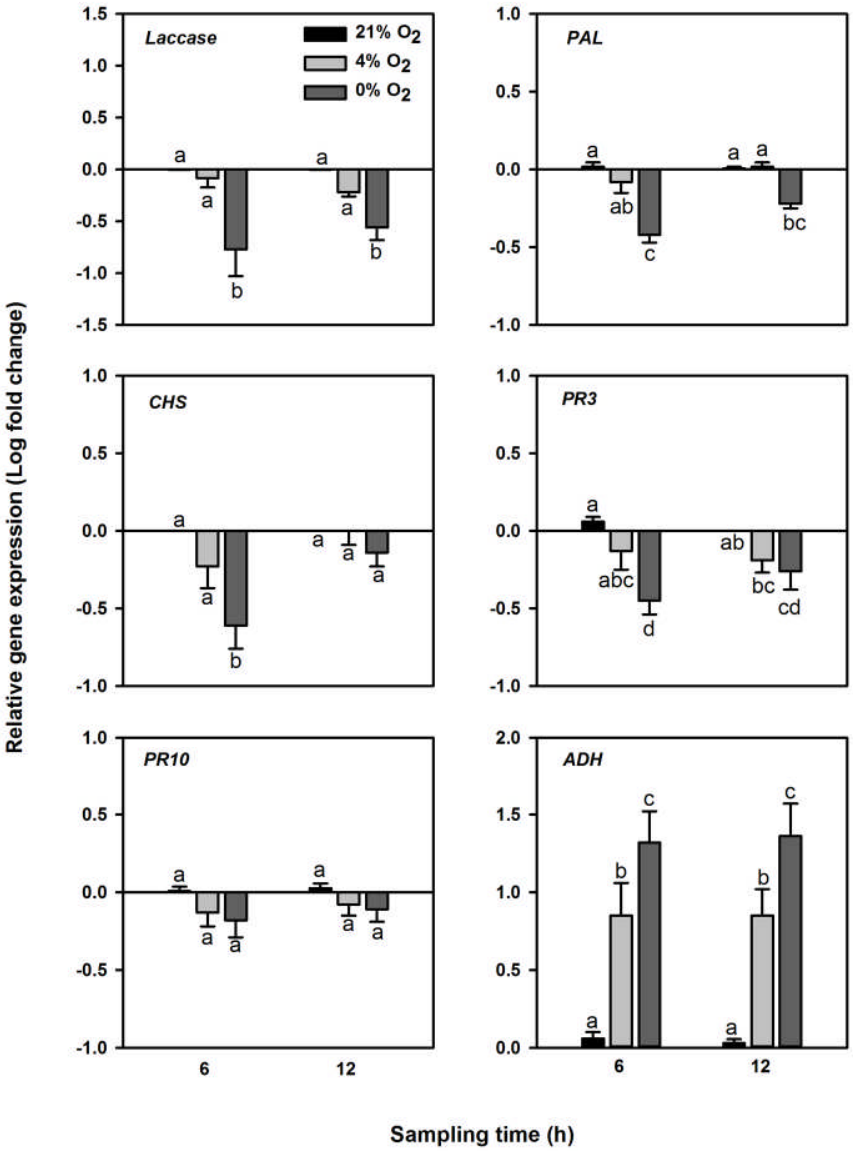


**Fig. 7.** Effect of oxygen level on the relative expression of soybean defense related genes in Fusarium virguliforme infected soybean roots, at 6 and 12 hours post inoculation (hpi). Beta actin was used as an internal control, and the normal oxygen treatment as the calibrator. Each treatment has three biological replicates and two technical replicates. Each bar represents the mean log fold-change of gene expression of three experimental runs, and the error bar indicates standard error of the mean (n=5), averaged over the two soybean cultivars MN1606 and Williams 82. Columns with different letters are significantly different ($P$<0.05).

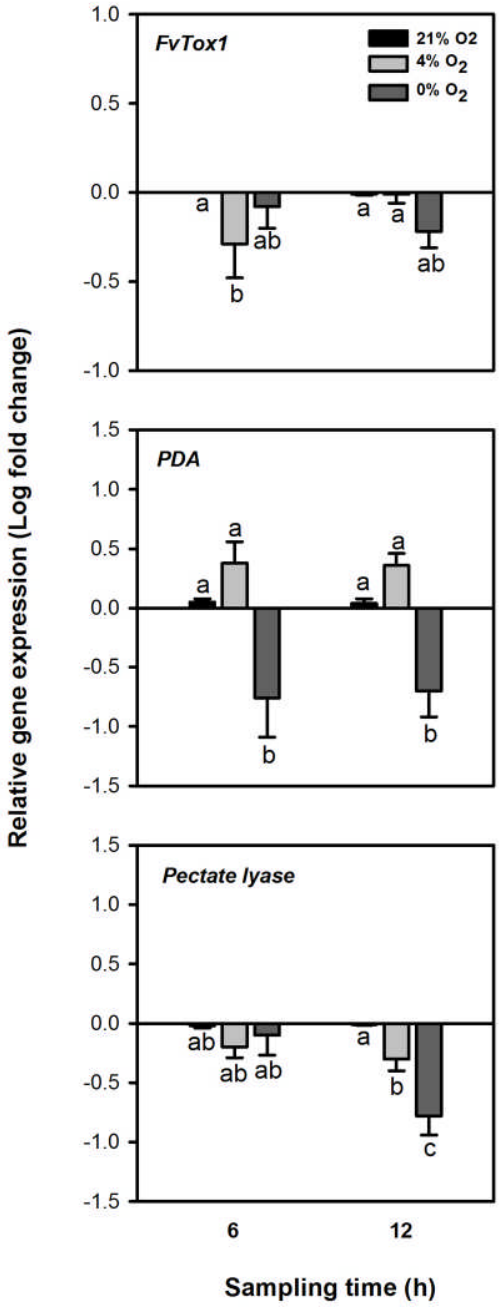


**Fig. 8.** Effect of oxygen treatments on the expression of *Fusarium virguliforme* pathogenicity genes in infected soybean roots, at 6 and 12 hours post inoculation (hpi). The 18s rRNA gene was used as an internal control and the normal oxygen treatment as the calibrator. Each treatment has three biological replicates and two technical replicates. Each column represents the mean log fold-change of gene expression of three experimental runs. Error bar indicates standard error of the mean (n=5). Columns with different letters are significantly different ($P$<0.05).

# CHAPTER 3.

# INDUCTION OF ETHYLENE BIOSYNTHESIS ENHANCES RESISTANCE AGAINST *FUSARIUM VIRGULIFORME*, THE CAUSAL AGENT OF SOYBEAN SUDDEN DEATH SYNDROME

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## Abstract

Ethylene is a gaseous hormone that regulates plant responses to biotic and abiotic stresses. To investigate the importance of ethylene in soybean resistance to *Fusarium virguliforme* (Fv), the causal agent of sudden death syndrome (SDS), soybean cultivars Williams 82 and MN1606 were treated 24 h before and 24h after *Fv* inoculation with either ethephon (ethylene inducer), cobalt chloride (ethylene biosynthesis inhibitor), or 1-MCP (ethylene perception inhibitor). Inoculated plants were grown for 21 days at 24°C in the greenhouse and then evaluated for SDS severity and expression of soybean defense genes. Plants treated with ethephon showed lower SDS foliar severity ($P<0.05$) compared to the other treatments, whereas those treated with cobalt chloride or 1-MCP showed the same or higher SDS foliar severity compared to the water-treated control.

Ethephon application resulted in activation of genes involved in ethylene biosynthesis, such as ethylene synthase (*ACS*) and ethylene oxidase (*ACO*), and genes involved in soybean defense response, such as pathogenesis-related protein (*PR*), basic peroxidase (*IPER*), chalcone synthase (*CHS*), and transcription factors. Cobalt chloride and 1-MCP treatments had little or no effect on these genes. Moreover, ethephon had an inhibitory effect on *in-vitro* growth of *Fv* on PDA media. Our results suggest that ethephon application inhibits SDS development directly by slowing *Fv* growth and indirectly by inducing soybean ethylene signaling and the expression of defense related genes.

## Introduction

Sudden death syndrome (SDS), caused by the soilborne fungus *Fusarium virguiforme* (*Fv*) (Aoki, O'Donnell, and Scandiani 2005) is one of the most damaging diseases to soybean production in North and South America. In the last two decades, SDS was ranked among the top ten most damaging soybean diseases in the united states, with average yield losses ranging from 0.3 to 2 million metric tons per year (Wrather and Koenning 2006; Leandro et al. 2013). As a soilborne pathogen, *Fv* infect roots at early soybean growth stages, causing root rot and reduction in root biomass. The fungus then releases phytotoxins that cause foliar interveinal chlorsis and necrosis and premature defoliation; these foliar symptoms usually appear during reproductive growth stages (Roy 1997; Leandro et al. 2012).

Host resistance is the most effective management practice against SDS. However, resistance to SDS is quantitative, i.e. is controlled by multiple genes, which adds complexity to plant breeders trying to accumulate numerous QTL into a single cultivar (Luckew et al. 2013).

Other management strategies such as crop rotation, tillage, and delayed planting date are often inconsistent and have limitations (Hartman et al. 2015).

Treatment of plants with synthetic chemical elicitors, such as hormones or their analogs, can induce resistance against a broad spectrum of plant pathogens, a phenomenon known as systemic resistance (Pieterse et al. 2014; Walters et al. 2006, 2013). Induction of systemic resistance is controlled by plant hormones, such as salicylic acid (SA), jasmonic acid, and ethylene (ET) (Glazebrook 2005). In general, SA is known to play an important role in activation of plant defense mechanisms against infection by biotrophic or hemibiotrophic pathogens, and is required for induction of systemic acquired resistance. In contrast, JA and ET play a crucial role in resistance against necrotrophic pathogens, and are required for induced systemic resistance (Glazebrook 2005; Pieterse et al. 2014).

Ethylene is a gaseous hormone involved in multiple plant growth and developmental processes, as well as response to biotic and abiotic stresses (Abeles et al. 1992; Broekaert et al. 2006). Several studies showed that ethylene has a role in the development of disease resistance, as it induces the expression of phytoalexins and PR genes (Ecker and Davis, 1987; Broekaert et al. 2006). However, ethylene signaling may act as a positive or negative regulators of disease resistance, depending on pathogen life style and plant species (Van Loon et al. 2006; Adie et al. 2007). For example, exogenous application of ethylene or ethephon (ethylene releasing substance) induces resistance against different pathogens, such as *Macrophomina phaseolina* in *Medicago truncatula* (Gaige et al. 2010), *Magnaporthe oryzae* in rice (Singh et al. 2004), *Phytophthora capsici* in habanero pepper (Nunez-Pastrana et al. 2011), and *Botrytis cinerea* in grapevine (Belhadj et al. 2008). Plant mutants impaired in ethylene perception have also shown enhanced disease susceptibility, as reported for ethylene-insensitive tobacco plants inoculated with non-

pathogenic soilborne fungi (Knoester et al. 1998), and in ethylene insensitive soybean mutants infected with *Sclerotinia sclerotiorum, Septoria glycines* and *Rhizoctonia solani* (Bent et al. 2006; Hoffman et al. 1999). In contrast, other studies showed that ethylene may act as a virulence factor and play a role in disease development (O'Donnell et al. 2003; Balaji et al. 2008). For instance, soybean ethylene insensitive mutants developed less severe symptoms in response *Pseudomonas syringae* pv *glycinea*, and *Phytophthora sojae* (Hoffman et al. 1999).

Genes involves in ethylene biosynthesis has been shown to be induced in response to soybean infection to *Fv,* using transcriptomic analyses (Radwan et al. 2011, 2013). However, it is not clear if this ethylene accumulation affected SDS resistance positively or negatively. In this study, we investigate the role of ethylene in the soybean-*Fv* interaction by manipulating ethylene accumulation and responses by the application of ethylene inducing and ethylene suppressing chemicals.

## Materials and Methods

### Plant material

Two soybean [*Glycine max* (L.) Merrill] genotypes, Williams 82 (susceptible to SDS) and MN1606 (resistant to SDS), were used in all experiments. Four seeds were sown 1 cm below the soil surface in 240 ml Styrofoam cups, then thinned to one seedling per cup after germination. The plants were incubated in a greenhouse bench at 24°C, with a 16-h photoperiod, watered as needed, and fertilized once a week.

**Pathogen culture**

*Fv* isolate NE-305 was used as the inoculum source in all experiments. A single-spore *Fv* isolate was collected from an infected plant in Nevada, IA in 2006, and maintained in potato dextrose agar (PDA) media for long-term storage. For inoculum preparation, 21-day-old cultures on PDA were flooded with 20 ml of sterile distilled water (SDW), the conidia were dislodged with a rubber policeman, and the suspension was filtered through a double layer of sterile cheesecloth. The spore concentration was then adjusted to $10^6$ conidial/ml using SDW. The conidial suspension was used to infest a sand and cornmeal mixture, following the procedure described by Munkvold and O'Mara (2002). A mixture of 1900 ml of sand, 380 ml of cornmeal and 110 ml of SDW was autoclaved in 20 cm X 30 cm bags (Fisher scientific, Pittsburg, PA) for 1 hour at 121°C, on two consecutive days. Each bag was then amended with either 2 ml of the $10^6$-conidia/ml suspension, or with 2ml of SDW for the controls. The bags were incubated in the dark at room temperature for 6 days with daily mixing by hand, to keep a uniform distribution of *Fv*.

**Effect of chemical treatment on soybean SDS in the greenhouse**

An experiment was conducted at the Iowa State University greenhouse facility to test the effect of ethephon, cobalt chloride, and 1-MCP treatment on SDS development. A factorial experiment consisting of two cultivars (Williams 82, and MN1606), and seven chemical treatments: ethephon (2-chloroethyl phosphonic acid, Sigma-Aldrish, St. Louis, MO, USA) at concentrations of 0.1, 1, and 4 mM, cobalt chloride (Fisher Scientific, Pittsburg, PA) at concentrations of 0.1, and 1 mM, and 1-MCP (Agro Fresh Inc, Philadelphia, PA) at a concentration of 1.32 g/L. Ethephon is an ethylene biosynthesis inducer, cobalt chloride is an ethylene biosynthesis suppressor, and 1-MCP inhibits ethylene perception (van Loon et al. 2006).

The experimental units were arranged in a randomized complete block design with a total of seven blocks one replication per each.

The chemical treatments were applied when soybean seedlings were at VC stage (first unifoliate). Ethephon and cobalt chloride were applied as a soil drench using 15 ml per pot, and 1-MCP was applied by spraying the leaves until runoff. Control plants were treated in the same way but applying a soil drench or spraying with SDW. All compounds were dissolved in SDW. Twenty-four hours after treatment application, seedlings were transplanted in *Fv* infested soil, prepared by mixing the sand-cornmeal *Fv* inoculum with pasteurized sand: soil mixture (2:1) at a ratio of (1:15) inoculum to sand-soil mixture (v/v). Twenty-four hours after transplanting, a second chemical treatment application was applied to each plant.

***Disease and plant growth assessments***. Twenty-one days after transfer (DAT) to *Fv* infested soil, plants were destructively sampled and the roots were thoroughly washed with running tap water to remove soil particles. For severity of root rot and foliar symptoms, seven replicate cups per treatment combination were visually assessed as the percent of root area showing brown or black discoloration and the percent of leaf area showing chlorosis and necrosis typical to SDS, respectively. Root and shoot lengths were measured on fresh plants, and dry weight of shoots and roots was measured after drying in at oven at 70°C for 48 h.

***Expression of ethyene pathway and defense-related genes***. To determine the effect of chemical treatments on soybean defense-related genes as listed in (Table 1), a factorial experiment consisting of two cultivars (Williams 82 and MN1606), and four chemical treatments: water, ethephon 4 mM, cobalt chloride 1 mM, and 1-MCP at a concentration of 1.32 g/L. The

experimental units were arranged in a randomized complete block with a total of five blocks each with two replications, and the experiment was conducted twice. The first run was conducted simultaneously with the disease experiment under the same conditions, and the second experiment was done separately. Total RNA was extracted from whole root tissue sampled at 0, 2, and 4 days after transfer to *Fv* infested soil (DAT) the roots were carefully rinsed with tap water to remove soil particles, immediately frozen in liquid nitrogen, and then stored in -80°C until use. Each sample consisted of two roots pooled from each of two cups (two replication cups per block).

For RNA extraction, root samples were ground in liquid nitrogen to a fine powder, then total RNA was extracted using RNeasy mini kit (Qiagen, Germantown, MD, USA). DNA was cleaned using RNase-free DNase I (Invitrogen, Carlsbad, CA, USA) following the manufacture's procedure. RNA quantity was determined using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). For cDNA synthesis, 0.5 µg of total RNA was reverse transcribed using SuperScript III and oligo-dT primer (Invitrogen, Carlsbad, CA, USA). The cDNA was then diluted 10 times to a final concentration of 2.5 ng/ µl. Real-time PCR was performed using the Perfecta SYBER Green fast mix (Applied biosystmes, Foster City, CA) and the iQ5 detection system (Bio-Rad, Hercules, CA, USA). The reaction mix consisted of 10 µl master mix, 0.5 µl reverse and forward primers (250 nM final concentration), 8 µl of diluted cDNA, and the final volume was adjusted to 20 µl with RNase DNase free water (Invitrogen, Carlsbad, CA, USA). The cycling protocol consisted of 3 min at 95°C, 40 cycles of 10 s at 95°C, 15 s at primer annealing temperature (Table. 1), and 30 s at 72°C. Melting curve data was collected to check for non-specific amplification and primer dimers. Each treatment had 4 or 5 biological replicates,

with two technical replicates. Relative gene expression was calculated using $2^{-\Delta\Delta ct}$ method (Livak et al. 2001) in which, the water treated control was used as the calibrator and beta actin as the reference gene.

***Fusarium virguliforme population in soil.*** *Fv* population in soil was quantified using dilution plating on selective medium (Rupe et al. 1997), for the cobalt chloride 1 mM, ethephon 4 mM and water control treatments only. There were three replicate samples per treatment combination, with each sample consisting of soil from two cups pooled together.
One gram of soil was placed in 100 ml of SDW in 250 ml flasks, and then 1 ml was transferred to a test tube with 9 ml of SDW to prepare a $10^{-3}$ dilution. The solution was thoroughly shaken and 0.2 ml was spread over modified Nash and Snyder's medium (MNSM) using a rubber policeman. The plates were then incubated at room temperature (24°C) for 5 days in the dark, and the numbers of colonies were counted to obtain the number of colony forming units per gram of soil (CFU/g soil).

**Effect of chemical treatment on the growth of *Fv in-vitro***

To investigate the effect of ethephon and cobalt chloride on *Fv* growth and development, full strength PDA media with antibiotics [tetrachlorocycline (0.15g/L) and streptomycin (0.15g/L)] was prepared and supplemented with ethephon or cobalt chloride. Solutions of ethephon and cobalt chloride were prepared from 10 mM concentration in which 100, 200, and 400 ml of stock solution were added to flasks containing PDA to complete the volume to one liter with a final concentrations of ethephon 1, 2, and 4 mM/L, respectively. For cobalt chloride 100, and 200 ml of stock solution were used. All chemicals were added to cool PDA media (v/v)

before agar solidification. A 4-mm diameter plug of a twenty-eight day-old *Fv* culture was placed, with the mycelium side facing down, in the center of petri dishes (100 mm X 15 mm) containing approximately 20 ml of amended PDA. A total of ten plates per treatment were arranged in a completely randomized design. Plates were incubated at room temperature (24°C) for 14 days under dark conditions. At the end of the incubation period, *Fv* colony diameter was measured in two perpendicular directions on each plate. Each plate was flooded with 5 ml of SDW, filtered with two layers of cheesecloth, and the conidial concentration was counted using a hemocytometer. The experiment was conducted three times.

**Data analyses**

For the greenhouse experiment, analysis of variance was performed using the PROC GLIMMIX procedure of SAS version 9.3 (SAS institute, Cary, NC) to determine the effects of treatments on root rot severity, foliar disease severity, *Fv* inoculum density in soil, gene expression, and root and shoot dry weight and length. Chemical application and cultivar were used as a fixed effect, and block and run were used as random effects.

For the *In-vitro* experiment, analysis of variance was performed using the PROC GLIMMIX procedure to determine the treatment effect of chemical on *Fv* colony diameter and number of conidia. Chemical application was used as a fixed effect, and replication and run were used as random effects. Fisher's protected least significant difference test ($P<0.05$) was used to detect the significant differences between treatments.

## Results

### Effect of chemical treatments on SDS and plant growth in the greenhouse

***Foliar and root rot severity.*** Chemical treatment and soybean variety significantly ($P<0.001$) affected SDS foliar symptoms and their interaction was significant. Therefore, the analysis was done separately by cultivar. In both soybean cultivars, plants drenched with ethephon at concentrations of 0.1, 1, or 4 mM exhibited a significant reduction in SDS foliar symptoms compared to the water treated control (Fig.1A). In contrast, plants sprayed with 1-MCP showed significant increases in SDS foliar symptoms compared to all treatments only in cultivar MN1606, whereas no significant difference was observed in cultivar Williams 82 (Fig. 1A). The application of a cobalt chloride drench at concentrations of 0.1 or 1 mM showed no significant effect on SDS foliar symptoms compared to controls. There were no significant differences in root rot severity between chemical treatments and the water control (Fig 1B).

***Root and shoot dry weight and length*.** There were significant chemical treatment and cultivar main effects in all growth parameters tested, and significant interactions between chemical treatment and cultivar; therefore, the analysis was conducted separately by cultivar. In cultivar MN1606, seedlings treated with ethephon at 0.1 mM showed higher shoot and root dry weights compared to the water control, whereas treatments with ethephon at 1 or 4 mM showed no effect on dry weights. Cultivar Williams 82 root dry weight did not differ significantly among treatments, whereas shoot dry weight was greater in seedlings treated with ethephon at 0.1 mM compared to ethephon at 4 mM. However, 0.1 and 4 mM ethephon tratments showed no

significant difference compared to the water control. Soybean seedlings treated with cobalt chloride or 1-MCP, showed no significant difference in root dry weight compared to control in both cultivars. Except in shoot dry weight of cultivar MN1606, where seedlings treated with 0.1 mM cobalt chloride showed higher dry weight compared to 1-MCP and control seedlings (Table 2).

Ethephon treatment significantly reduced root length compared to the control when applied at a rate of 4mM in cultivar MN1606, and at a rate of at 0.1 mM in cultivar Williams82. For shoot length, seedlings treated with ethephon at 1mM showed greater shoot length compared to the control in cultivar MN1606, but no treatment effects were observed in Williams 82. Cobalt chloride and 1-MCP treatments did not affect root or shoot length, except in cultivar Williams 82, where seedlings treated with cobalt chloride at 1 mM showed greater shoot length compared to MCP and the control (Table. 2).

**Activation of ethylene signaling pathway in response to ethephon treatment**

There was a significant main effect of chemical treatment at all time points tested, and a significant interaction chemical treatment and cultivar; therefore analysis was conducted separately by cultivar. In order to investigate the effect of ethphon on ethylene biosynthesis, the expression of ACS, ACO, and PR2 genes were tested. In both soybean cultivars, and at all time points, ethephon application significantly induced expression of the *PR2* gene, while cobalt chloride and 1-MCP applications showed no effect on the expression of this gene compared to the control. For the ACS gene, both cultivars showed the highest expression level in response to ethephon treatments compared to the other treatments at 0 DAT. At 2 and 4 DAT, the same pattern was observed in cultivar MN1606, but in Williams 82 there were no significant treatment

differences at 2 DAT, and the expression was higher in 1-MCP, compared to ethephon and control, at 4 DAT. For the ACO gene, in cultivar MN1606, expression was higher in ethephon treated seedlings compared to cobalt chloride and control at 2 and 4 DAT, but no significant difference was observed among treatments at 0 DAT. In cultivar Williams 82, *ACO* expression was higher in response to ethephon treatment compared to control at 0 and 2 DAT, but ethephon treated seedlings showed the lowest *ACO* expression at 4 DAT (Fig. 2).

**Effect of ethephon treatment on soybean defense-related genes in response to *Fv* infection**

There was a significant main effect of chemical treatment at all time points tested, and a significant interaction chemical treatment and cultivar; therefore analysis was conducted separately by cultivar. In both soybean cultivars, the expression levels of *PR1* and *PR3* genes were significantly higher in ethephon-treated seedlings compared to 1-MCP and control treatments at 0 DAT. The expression level of the *PR10* gene was 3-fold higher in ethephon-treated seedlings compared to controls in MN1606, but did not differ among treatments in Williams 82. At 2 and 4 DAT, all three *PR* genes studied were highly expressed in response to ethephon-treated roots compared to all other treatments in both cultivars, except at 4 DAT in Williams 82 where no significant difference was observed among treatments in *PR10* gene expression. Cobalt chloride and 1-MCP treatments showed no effect on the expression of *PR* genes compared to control, in either cultivar and at any time point (Fig. 3 & 4). Furthermore, at 2 DAT, genes involved in ethylene biosynthesis and pathogenesis related-proteins, such as *ACO, ACS, PR1, PR2,* and *PR10*; showed higher expression levels in the resistant cultivar MN1606 compared to the susceptible cultivar Williams 82, whereas at 0, and 4 DAT no difference among cultivars was observed.

*CHS* expression was highest in ethephon treated seedlings compared to the other treatments at 0 and 2 DAT in cultivar MN1606, although the expression level returned back to the control levels at 4 DAT. In cultivar Williams 82, a similar increase in CHS expression in response to ethephon was observed at 2 DAT, but there were no significant differences among treatments at 0 and 4 DAT. The *IPER* gene expression was highest in response to ethephon treatment compared to all other treatments, at all time points, and in both soybean cultivars, except at 4 DAT in cultivar Williams 82 where no significant difference was observed. At all time points, ethephon treatment had no effect on the expression level of *ERF* gene compared to control or cobalt chloride treatments, except in cultivar Williams 82 at 4 DAT, in which the highest expression level of *ERF* gene was observed in ethephon treated seedlings. In contrast, 1-MCP treated seedlings showed consistently lower level of *ERF* expression compared to ethephon treatment at all time points in both cultivars (Fig. 3 & 4).

**Effect of chemical treatment on *in-vitro Fv* growth**

***Colony growth and sporulation.*** Analysis of variance showed a significant effect of chemical treatment on Fv colony diameter and conidation. *Fv* colony diameter was significantly reduced in media amended with ethephon compared to un-amended media (Fig. 5A), Colony morphology was also affected by ethephon; colonies on PDA supplemented with ethephon showed irregular shape and developed a purple to pinkish color that became more pronounced as the ethephon concentration increased. *Fv* colonies grown on PDA supplemented with cobalt chloride showed normal growth rate and colony morphology compared to the control (Fig. 5A).

All ethephon concentrations significantly reduced the number of *Fv* conidia produced in each colony compared to the water control. Colonies grown in media amended with cobalt chloride at 0.1 mM showed the same number of conidia as controls, whereas amendment with cobalt chloride at 1 mM reduced conidiations compared to the control (Fig. 5B). However, chemical treatments did not affect conidial germination.

## Discussion

In this work, we investigated the effect of ethylene suppression and induction on soybean resistance against *Fv* infection. Previous studies have shown the importance of phytohormones signaling in resistance against plant diseases (Bari and Jones 2009; Robert-Seilaniantz et al. 2011), but to our knowledge, this is the first report on the role of the ethylene hormone in the soybean-*Fv* interaction. We showed that induction of ethylene biosynthesis in soybean roots using a soil drench with ethephon reduced SDS foliar symptoms development by up to 75% compared to a drench with water. In contrast, suppression of ethylene biosynthesis or perception, by application of cobalt chloride or 1-MCP, respectively, resulted in the same or higher SDS development compared to the control, respectively. Furthermore, a direct inhibitory effect of mycelium growth and sporulation was observed on *Fv* plugs grown on PDA media supplemented with ethephon, whereas media amendment with cobalt chloride either had no effect or reduced Fv growth or sporulation compared to controls. At the molecular level, drench applications of ethephon at 4mM enhanced the expression of genes involved in soybean ethylene biosynthesis and defense responses. Taken together, these results suggest that ethephon soil application is a promising inducer of ethylene signaling pathway that may play a positive role in soybean resistance against *Fv* infection.

Our findings showing reduction of SDS severity in response to exogenous application of ethephon induction of ethylene signaling are in agreement with previous studies in other pathosystems (van Loon et al. 2006). For example, pretreatment of soybean seedlings with ACC (ethylene precursor) enhanced plant survival rate against *Phytopthora sojae* infection (Sugano et al. 2013). Similarly, ethephon treatment triggered protection of grapevine leaves against *Erysiphe necator*, the causal agent of powdery mildew (Belhadj et al. 2008), and transgenic rice with inducible ethylene production was more resistant to *Magnaporthe oryzae* and *Rhizoctonia solani* (Helliwell et al. 2013). Furthermore, ethylene insensitive soybean lines were more susceptible to white mold caused by *Sclerotinia sclerotiorum* compared to wild type in field conditions (Bent et al. 2006). Despite abundant evidence for enhanced disease resistance in response to ethylene, other studies showed that ethylene might act as a negative regulator of disease resistance (Scherm et al. 1998; Itai et al. 2012; Robison et al. 2001; Lu et al. 2014) growth, or by both actions (Nunez-Pastrana et al. 2011).

In our study, *Fv* cultures grown on PDA media supplemented with ethephon showed reduced colony size, reduced sporulation and different morphology compared to cultures grown in non-amended media. This result is consistent with a report of delayed growth of *Phythophthora capsici*, the causal agent of Phytopthora blight in bell pepper, on PDA media supplemented with 5mM ethephon (Nunez-Pastrana et al. 2011). However, in contrast to the inhibitory effect observed *in-vitro*, ethephon had no effect on *Fv* population in soil (data not shown).

It has been well documented that plants defend themselves against pathogen attack by inducing defense responses that are modulated by phytohormones (Bari and Jones 2009).

However these hormone-regulated defenses are often accompanied by fitness costs (Edelman et al. 2014; Denancé et al. 2013). For example, Arabidopsis mutant *cev1* that constitutively expresses the jasmonic acid and ethylene pathways was resistant against powdery mildew, but also showed a stunted phenotype (Ellis and Turner 2001). In our study, application of ethephon affected root and shoot dry weight and length, however, this effect was dependent on cultivar and dose. For example, in cultivar MN1606, the greatest shoot and root dry weight were observed at the lowest ethephon concentration, whereas no effect was observed among the other concentrations or in cultivar Williams 82. Also, root length was decreased by some ethephon treatments in both cultivars. This is consistent with a study done by Urwiler and Stutte (1986) who found that application of ethephon at high rate affected soybean seed and pod development, whereas low rate application had no effect.

Furthermore, in our study we found that low cobalt chloride at concentrations of 0.1 or 1 mM did not affect soybean growth compared to the control, whereas a phytotoxic effect was observed on leaves treated with high concentration of cobalt chloride 10 mM (data not shown). These results were consistent with earlier studies (Jayakumar and Jaleel 2009; Jaleel et al. 2009; Vijayarengan et al. 2009) showing that lower cobalt (50mg/kg) can increase soybean growth and nodulation, while high doses adversely affect these parameters.

At the molecular level, manipulation of the ethylene pathway using pharmacological or genetic approaches induced the expression of plant defense-related genes and enhanced resistance against different biotic and abiotic stresses (Van Loon et al. 2006; Kazan 2015). For instance, exogenous application of ethephon enhanced resistance to charcoal rot in *Medicago truncatula* (Gaige et al. 2010), *Phytopthora sojae* in soybean (Sugano et al. 2013), *Phytophthora capsici* in Habanero pepper (Nunez-Pastrana et al. 2011), and *Erysiphe necator* in grapevine (Belhadj et al.

2008). This increased resistance was probably due to accumulation of pathogenesis-related proteins and antimicrobial compounds such as phytoalexins (Broekaert et al. 2006). Furthermore, ethylene insensitivity in tobacco impaired resistance against soilborne fungi (Knoester et al. 1998) and an ethylene insensitive pea mutant *ein2* developed more severe symptoms when challenged by *Fusarium oxysporum* or *Pythum irregulare* compared to wild type peas (Foo et al. 2016; Blake et al. 2015). Similarly, our study showed that induction of ethylene signaling in soybean roots using ethephon enhanced resistance against SDS foliar symptoms and increased accumulation of key defense response genes such as *CHS,* and basic peroxidase *IPER,* compared to water treated control.

Ethephon application also induced the expression of various pathogenesis related-proteins (PR) genes such as *PR1, PR2, PR3,* and *PR10* that are encode enzymes with antifungal activity, as previously reported (Shrestha et al. 2008; Mauch et al. 1992). The *PR1* gene is commonly used as a marker for salicylic acid signaling and systemic acquired resistance (van Loon and van Strien 1999). In agreement with our results, Nunez-Pastrana et al (2011) showed a direct correlation between the survival of ethephon-treated Habanero pepper seedlings against *Phytophthora capsici* and the accumulation of the *PR1* gene. The *PR2* and PR3 genes code for a β-1,3 endoglucanase and a class I chitinase, respectively; these enzymes induce plant defenses by hydrolyzing fungal cell wall components such as β-1,3-glucans and chitin, respectively (Balasubramanian et al. 2012; Grover 2012). Transgenic Indian cotton also expressed a rice chitinase gene that conferred resistance to *F. oxysporum* and *Alternaria macrospora* infection (Ganesan et al. 2008; 2009). Furthermore, ethylene pretreatment increased *PR2* and *PR3* activity and induced resistance against *Botrytis cinerea* in tomato and *Phytophthora megasperma* in soybean (Takeuchi et al. 1990; Díaz et al. 2002).

Another important host defense mechanism involves the rapid accumulation of reactive oxygen species (ROS) in response to pathogen attack, a phenomenon called oxidative burst (Mittler et al. 2004). This reaction is directly toxic to pathogens and can lead to a hypersensitive response that prevents further pathogen spread. In our study, we did not measure ROS levels but we quantified the expression of the peroxidase gene *IPER*. Peroxidases are involved in ROS regulation, cell wall lignification, and in defense response against pathogen attack (Passardi et al. 2005). Our data showed a strong induction of *IPER* 24 hours after ethephon application compared to the water-treated control. Furthermore, the expression of this gene was approximately seven times greater in the SDS resistant cultivar compared to the susceptible one. This result is in agreement with Yi and Hwang (1998) who demonstrated that *IPER* accumulated in soybean roots in response to ethephon treatment, and that *IPER* accumulation was greater in soybean hypocotyls infected with an incompatible race of *P. sojae* compared to low levels in the compatible interaction.

In contrast to the positive role of ethylene induction on resistance against SDS, suppression of ethylene biosynthesis by cobalt chloride had no effect on SDS foliar development. However, in one of the two cultivars tested, blocking of ethylene perception by 1-MCP resulted in more severe SDS symptoms compared to water or ethephon-treated seedlings, suggesting a possible role of ethylene perception in resistance against *Fv* infection. Similarly in tomato, silencing of multiple *ACS* genes that are involved in ethylene biosynthesis did not affect *Mi-1*-mediated resistance against root-knot nematode infection. However, the tomato Never ripe (*Nr*) mutant that is compromised in ethylene perception was more attractive to the infective juveniles and showed enhanced susceptibility to RKN infection (Mantelin et al. 2013; Fudali et al. 2012).

Another possible explanation for the minor effect of ethylene biosynthesis suppression on SDS development could be that one or more additional signaling pathways might interact with ethylene to regulate soybean defense response against *Fv* infection. The defense response against necrotrophic pathogens is usually regulated by a synergistic interaction between the ethylene and jasmonic acid signaling pathways (Glazebrook 2005). Transcriptome analysis of *Arabidopsis thaliana* showed that half of the genes that were induced by ethylene were also induced by jasmonic acid treatment (Schenk et al. 2000). A study at Iowa State University by the Whitham lab (personal communication) showed that silencing of key genes in ethylene or jasmonic acid signaling pathways enhanced SDS symptoms development.

In conclusion, the results of this study support our hypothesis that the ethylene-signaling pathway is important in resistance against SDS. We observed a correlation between ethylene biosynthesis, accumulation of defense-related genes, and SDS resistance in response to ethephon treatment. A limitation of our study is that ethephon was applied to soybean seedlings at VC stage, before *Fv* infection, and under controlled environmental conditions. Future work should validate the effect of ethephon on SDS under field conditions and at different application times. If ethylene is shown to enhance resistance against *Fv* infection in field conditions, then transcriptomic analysis of soybean seedlings in response to ethephon treatment is needed to identify resistant genes that could be incorporated into breeding against *Fv*.

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**Table 1. Oligonucleotide primers used for quantitative real-time PCR**

| Gene Name | Sequence (5 to 3) Forward | Reverse | Descripiton | References |
|---|---|---|---|---|
| PR1 | TGTGTTGTGTTTGTTAGGGTTAGTCA | TGTTGGTGAGTCTTGAGCATACG | PR1a precursor antimicrobil protein | (Zhong et al. 2014) |
| PR2 | GTCTCCTTCGGTGGTAGTG | ACCCTCCTCCTGCTTTCTC | Beta-1,3-Endoglucanase | (Zhong et al. 2014) |
| PR3 | GCACTTGGTCTGGATTTG | GGCTTGATGGCTTGTTTC | Chitinase class I | (Zhong et al. 2014) |
| PR10 | GCCCAGGAACCATCAAGAAG | CGCTGTAGCTGTATCCCAAG | Intercellular pathogensis related protein 10 | (Sugano et al.2013) |
| CHS | AGGCTGCAACTAAGGCAATC | TAATCAGCACCAGGCATGTC | Chalcone synthase | (Zhong et al. 2014) |
| IPER | CTCTCAGGTGCTCATACATTCG | TGGATCAGGTTTGCCAGTTC | Basic peroxidase | (Zhong et al. 2014) |
| ERF | GCTTAAGGAGATGAACTATGCAAA | TTGACGCTAATTTTCCTTCTCAA | Ethylene elongation factor 1 | (Sugano et al.2013) |
| ACO | CATGTTTTTCGCGTTCTCCT | AAGTACAGAAAGAAAGGGATGGA | 1-aminocyclopropane-1-carboxylic acid oxidase | (Sugano et al. 2013) |
| ACS | CTTAGGCTCAGTTTCTCTTCAAGGATATTTGAT | CGCTCGAGTAGAACCCAGATCCAATC | 1-aminocyclopropane-1-carboxylic acid synthase | (Tucker et al. 2010) |
| Actin | TCCAAGGGGACCTAACGGAGA | TGGGTCAAGAGCTGGATGGTG | Soybean beta actin | (Radwan et al. 2011) |

**Table 2.** Effect of chemical treatment on soybean growth and development

| | Growth parameter [a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | RDW [b] | | SDW | | RL | | SL | |
| Cultivar [c] | R | S | R | S | R | S | R | S |
| Treatment [d] | | | | | | | | |
| Control | 0.24 b | 0.22 a | 0.39 bc | 0.52 ab | 15.19 a | 13.86 ab | 12.41 b | 8.78 b |
| E0.1 | 0.31 a | 0.22 a | 0.50 a | 0.57 a | 13.3 ab | 12.84 abc | 13.36 ab | 9.7 ab |
| E1 | 0.25 ab | 0.22 a | 0.42 abc | 0.52 ab | 13.9 a | 11.75 c | 15.10 a | 9.46 ab |
| E4 | 0.22 b | 0.20 a | 0.36 c | 0.47 b | 12.8 b | 12.38 bc | 12.75 ab | 9.7 ab |
| CC0.1 | 0.26 ab | 0.22 a | 0.50 a | 0.50 ab | 15.25 a | 13.31 ab | 13.87 ab | 8.75 b |
| CC1 | 0.25 ab | 0.24 a | 0.47 ab | 0.59 a | 14.9 ab | 13.95 a | 12.87 ab | 10.86 a |
| 1-MCP | 0.20 b | 0.22 a | 0.41 abc | 0.53 ab | 13.8 ab | 13.90 ab | 12.87 ab | 9.03 b |

[a] Growth parameter: RDW= root dry weight, SDW= shoot dry weight, RL= root length, and SL= shoot length

[b] data represents the mean of fourteen replications (seven replications X two runs). Numbers followed by different letters within the same column are significantly different at $P<0.05$ according to Fisher's least significant difference test.

[c] Cultivar: R= resistant cultivar MN1606, S= susceptible cultivar Williams82

[d] Treatment: Control = water treated, E0.1= ethephon 0.1 mM, E1= ethephon 1 mM, E4= ethephon 4 mM, CC0.1= cobalt chloride 0.1 mM, CC1= cobalt chloride 1 mM, and 1-MCP= methylcycloprope

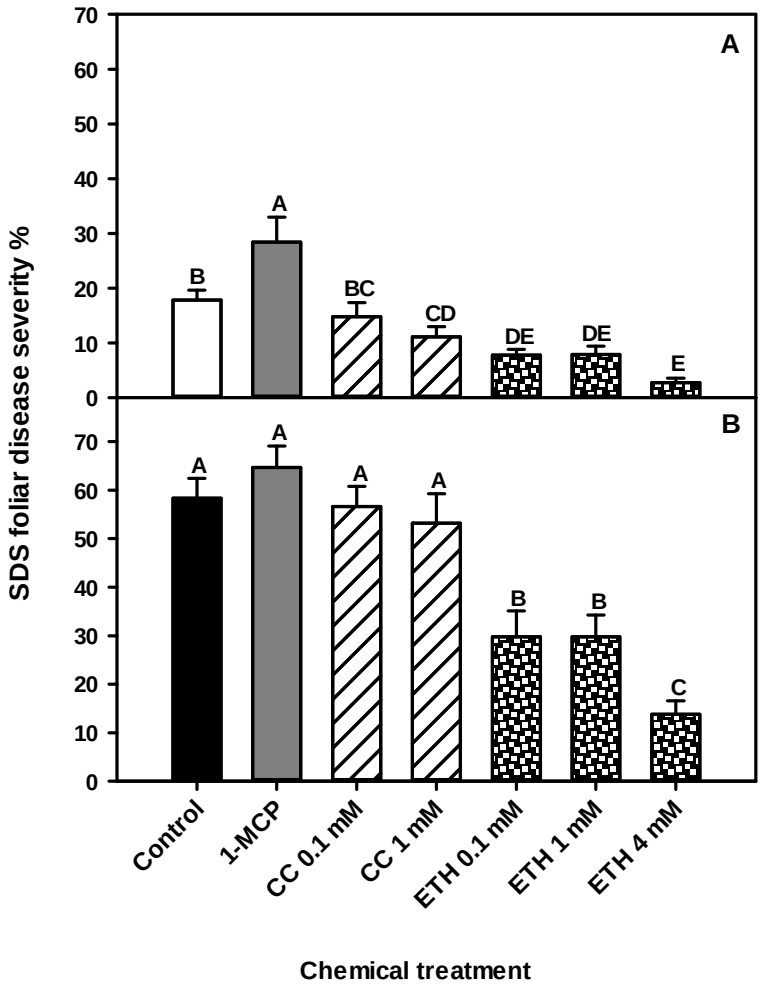


**Fig. 1.** Effects of chemical treatments on severity of foliar symptoms of soybean sudden death syndrome in resistant cultivar MN106 (A) and susceptible cultivar Williams 82 (B). Soybean seedlings were drenched with water (control), ethephon (ethylene inducer) at concentrations of 0.1 mM, 1 mM, and 4 mM, or cobalt chloride (ethylene suppressor) at concentrations of 0.1 mM, and 1 mM, 24 h before and 24 h after transplant into soil infested with *Fusarium virguliforme*. To 1-MCP (ethylene perception suppressor) was sprayed until run off at the same times. SDS symptoms were assessed 21 days post inoculation (DAT). Each bar represents the mean of 21 replicates (7 cups x 3 runs), and the error bar represents the standard error of the mean. Means with different letters are significantly different ($P$<0.05).

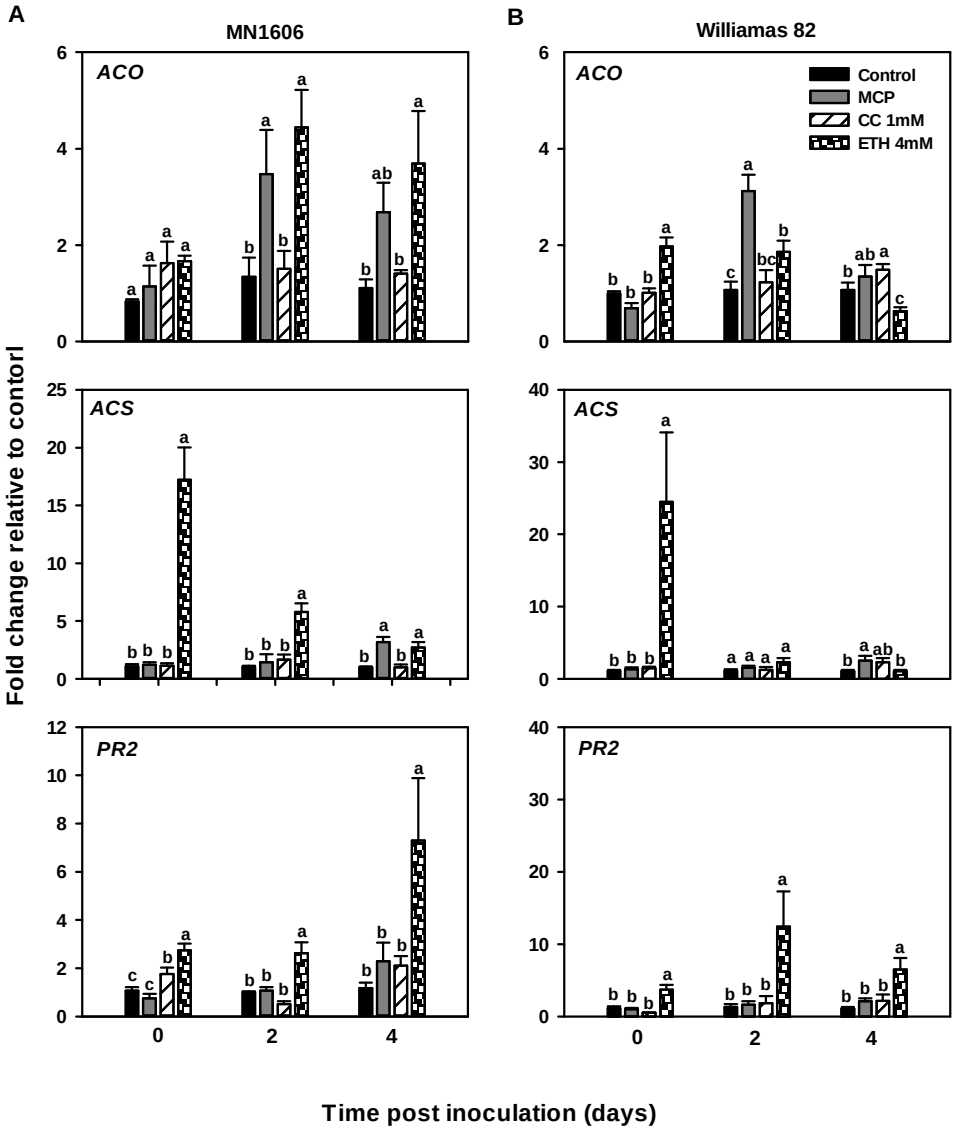


**Fig. 2.** Effect of chemical treatments on the expression of soybean ethylene biosynthesis and signaling genes in resistant and susceptible cultivars, MN1606 (A) and Williams82 (B), respectively. Soybean seedlings were drenched with water (control), ethephon (ethylene inducer) 4 mM, or cobalt chloride (ethylene suppressor) 1 mM, 24 hours pre and post transplant into soil infested with *Fusarium virguliforme*. Roots were sampled at 0, 2, and 4 days after transplant. Plants sampled at time 0 received one chemical treatment, whereas those sampled at time 2 and 4 received two chemical treatments. The soybean actin gene was used as an internal control and the fold change was calculated by calibrating data to the infected water treated plants. Each treatment has four biological replicates and two technical replicates. Each column represents the mean log fold-change of gene expression of three experimental runs. Error bar indicates standard error of the mean (n=8). Columns with different letters are significantly different ($P$<0.05).

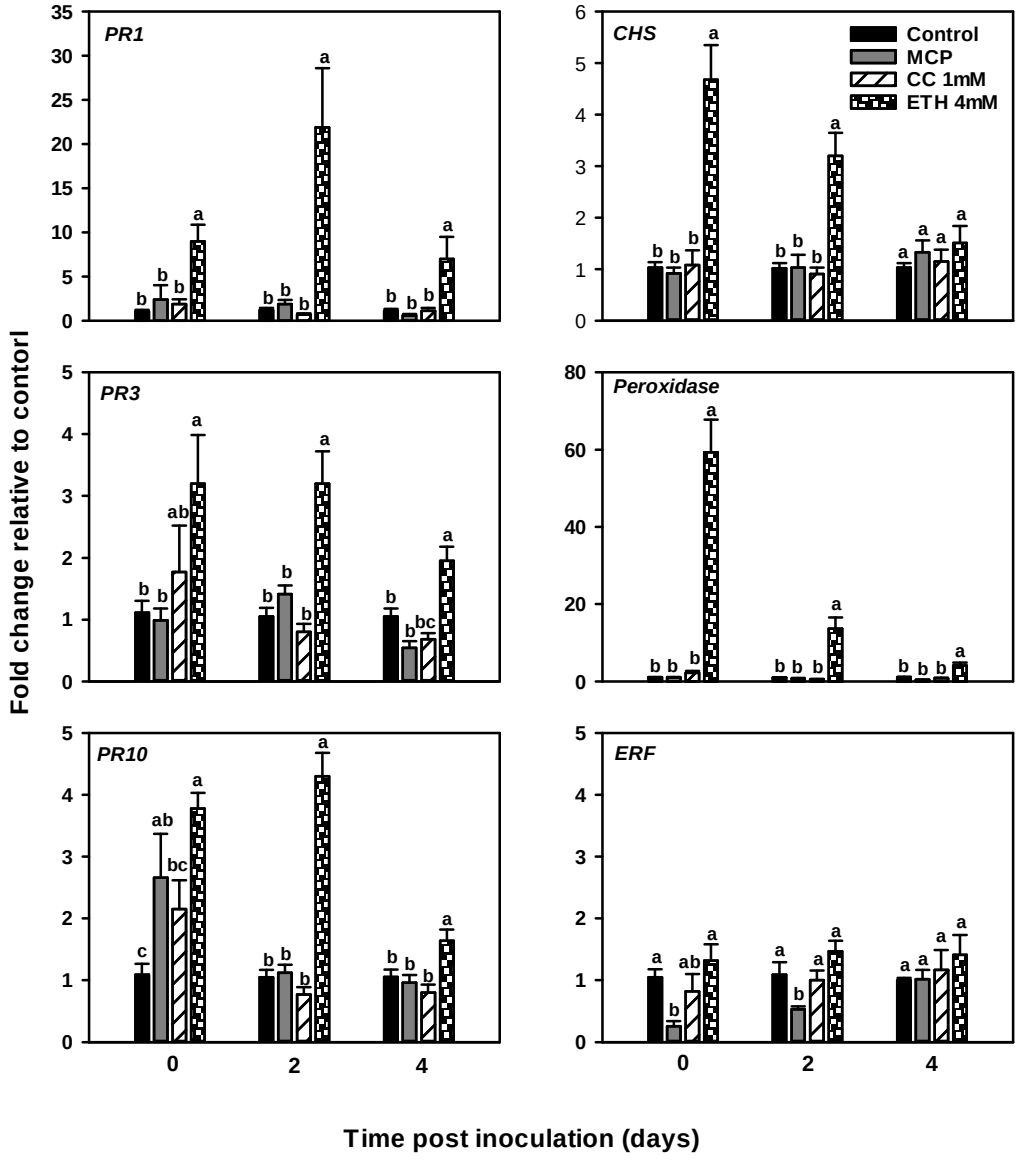


**Fig. 3.** Effect of chemical treatments on the expression of soybean defense-related genes in resistant cultivar MN1606. Soybean seedlings were drenched with water (control), ethephon (ethylene inducer) 4 mM, or cobalt chloride (ethylene suppressor) 1 mM, 24 hours pre and post transplant into soil infested with *Fusarium virguliforme*. Roots were sampled at 0, 2, and 4 days after transplant. Plants sampled at time 0 received one chemical treatment, whereas those sampled at time 2 and 4 received two chemical treatments. The soybean actin gene was used as an internal control and the fold change was calculated by calibrating data to the infected water treated plants. Each treatment has four biological replicates and two technical replicates. Each column represents the mean log fold-change of gene expression of three experimental runs. Error bar indicates standard error of the mean (n=8). Columns with different letters are significantly different ($P$<0.05).

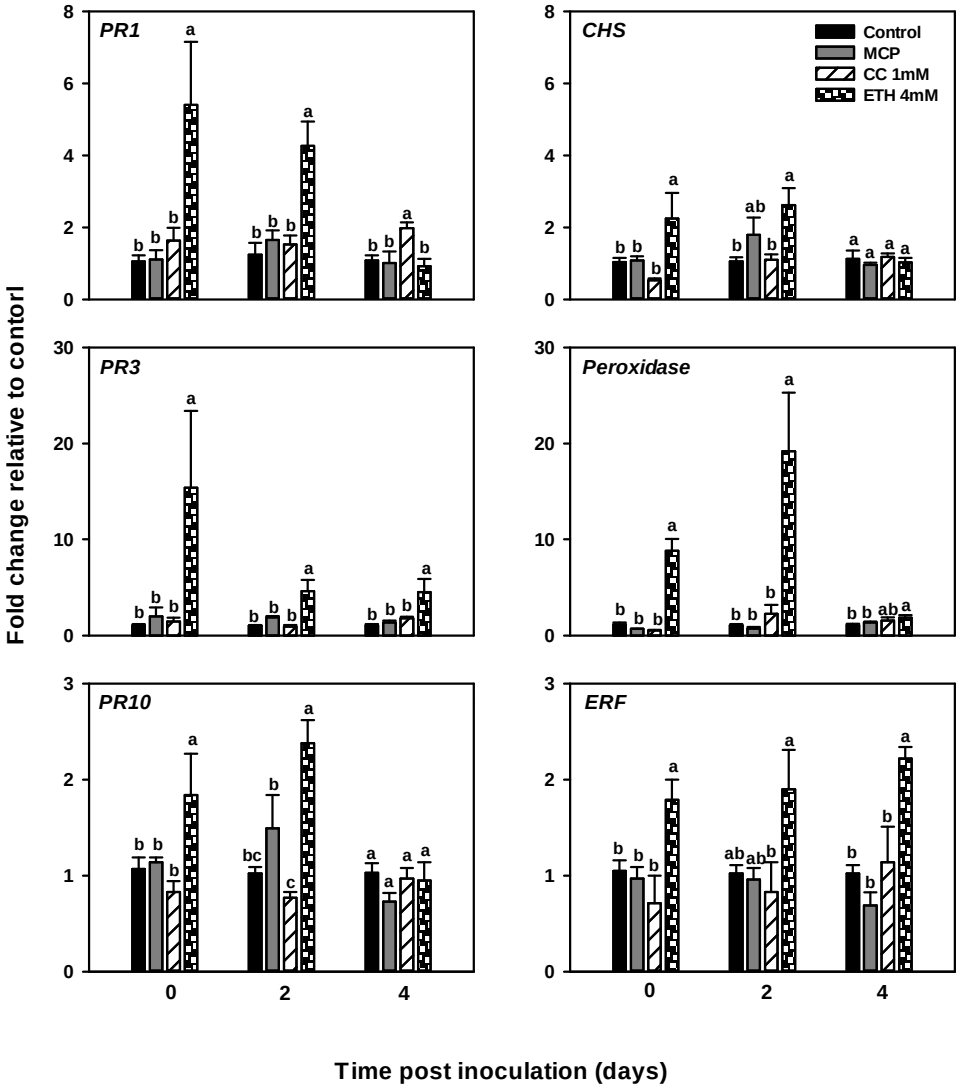


**Fig. 4.** Effect of chemical treatments on the expression of soybean defense-related genes in susceptible cultivar Williams82. Soybean seedlings were drenched with water (control), ethephon (ethylene inducer) 4 mM, or cobalt chloride (ethylene suppressor) 1 mM, 24 hours pre and post *Fusarium virguliforme* inoculation. Roots were sampled at 0, 2, and 4 days after *F. virguliforme*. Plants sampled at time 0 received one chemical treatment, whereas those sampled at time 2 and 4 received two chemical treatments. The soybean actin gene was used as an internal control and the fold change was calculated by calibrating data to the infected water treated plants. Each treatment has four biological replicates and two technical replicates. Each column represents the mean log fold-change of gene expression of three experimental runs. Error bar indicates standard error of the mean (n=8). Columns with different letters are significantly different ($P$<0.05).

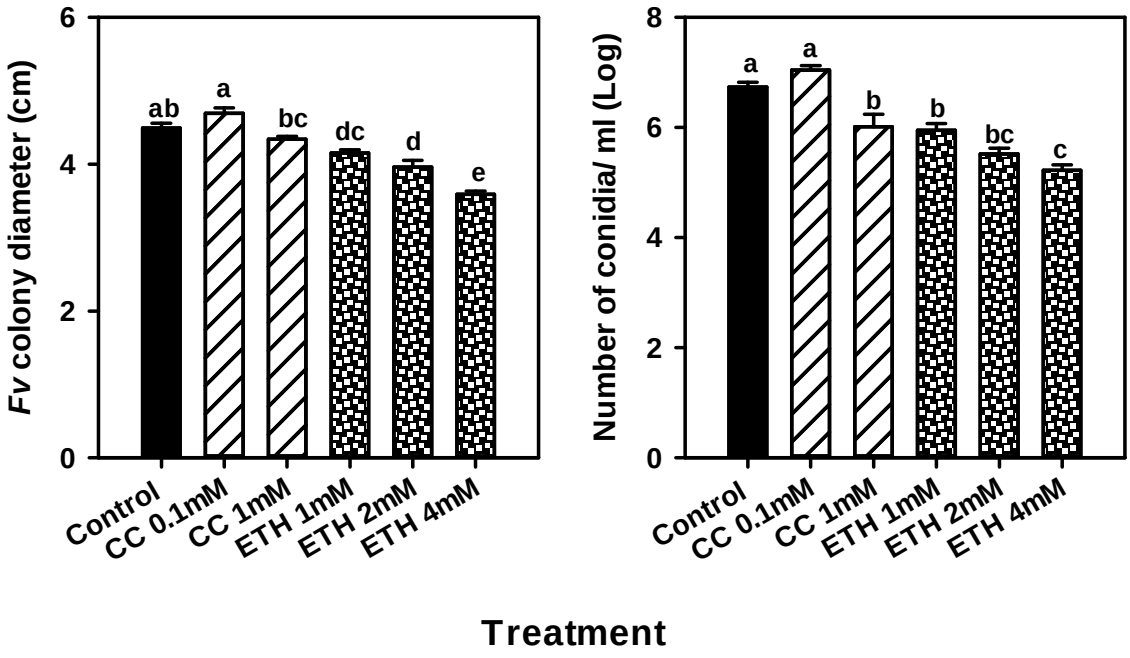


**Fig. 5.** Effects of ethephon and cobalt chloride on in mycelial growth *of Fusarium virguliforme* Mycelia plugs were grown for fourteen days on PDA plates containing water, ethephon (1mM, 2mM, and 4mM), or cobalt chloride (0.1mM, and 1mM). Colony diameter (A), and number of conidia per ml (B) were measured fourteen days after incubation at room temperature under dark conditions. Each bar represents the mean of 30 replicates (10 plates x 3 runs) and the error bar represents the standard error of the mean. Means with different letters are significantly different ($P<0.05$).

# CHAPTER 4.

## INFLUENCE OF ETHEPHON APPLICATION TIMING ON SUPPRESSIN OF SOYBEAN SUDDEN DEATH SYNDROME UNDER GREENHOUSE AND FIELD CONDITIONS


### Abstract

Soybean sudden death syndrome (SDS), caused by *Fusarium virguliforme* (*Fv*) is among the top five most important soybean diseases in the United States. Disease management primarily relies on genetic resistance and, more recently, seed treatments. The incorporation of plant defense inducers has not been investigated as an alternative or complementary management tool. The objective of this study was to understand the effect of ethephon (ethylene releasing compound) application at different soybean growth stages on SDS. In a greenhouse experiment, the following ethephon (0.1mM) treatments were compared: i) soil drench at planting (VP), ii) soil drench at VP followed by a second application at emergence (VP+VE), iii) soil drench at VP and unifoliate stage (VP+VC), and iv) soil drench at VP and at first trifoliate stage (VP+V1-V2). In a field study, the following ethephon (0.1 mM) application regimes were compared: i) soil drench (in-furrow) at planting, ii) soil drench at emergence (VE), and iii) foliar spray at V1-V2. Under greenhouse conditions, all ethephon treatments significantly reduced SDS foliar symptom severity by 50-60% compared to the untreated control ($P<0.05$) in the susceptible cultivar Williams82. However, SDS severity did not differ among ethephon treatments, showing that the second application did not enhance disease suppression compared to a single application at planting.

In the field, ethephon application at planting or at plant emergence significantly ($P<0.05$) reduced SDS foliar severity compared to the untreated control in 2015, but yield did not differ among treatments. In 2016, ethephon applications did not affect SDS or yield. These results suggest that the use of plant defense inducers, such as the product ethephon, should be further researched as a tool to help manage SDS under field conditions.

## Introduction

Sudden death syndrome (SDS), caused by *Fusarium virguliforme* (Aoki et al. 2003), ranks among the top five most damaging soybean [*Glycine max* (L.) Merrill] diseases in the United States ( Wrather et al. 2010, Wrather and Koenning 2006, 2009). Depending on environmental conditions, soybean cultivar, planting date, and time of disease establishment, yield loss might range from slight to more than 80% (Roy et al 1997). Cool, wet soil early at the growing season is favorable for the fungus to colonize soybean roots, causing rot and biomass reduction (Scherm and Yang 1996). The fungus also produces phytotoxins, causing foliar symptoms that usually appear at reproductive stages. The above-ground symptoms consist of interveinal chlorosis and necrosis, premature defoliation, and pod mottling (Roy et al. 1997).

The use of genetic resistance is the most effective strategy to manage SDS. However, breeding for SDS resistance is challenging due to the quantitative nature of the resistance and the strong effect of environmental conditions on the disease development (Luckew et al. 2013). Cultural practices, such as crop rotation, tillage, planting date, and control of soybean cyst nematode have been investigated as management options, but are not consistently

effective (Hartman et al. 2015). A fungicide seed treatment has recently been shown to suppress SDS and protect yield in field conditions (Weems et al. 2015; Kandel et al. 2016), but continued use of fungicides could result in the development of resistance in pathogens (Price et al. 2015; Matthiesen et al. 2015).

Another possible approach for SDS management could be the activation of plant defense mechanisms, either locally or systemically, using beneficial microorganisms or chemical elicitors, a phenomena known as induced resistance (Walters et al. 2013a; Thakur and Sohal 2013). Induced resistance is highly regulated by phytohormones, such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA) (Robert-Seilaniantz et al. 2011). In general, SA plays an important role in activation of plant defense mechanisms against infection by biotrophic or hemibiotrophic pathogens that need live tissue to obtain nutrients and complete their lifecycle (Glazebrook 2005). Furthermore, SA is required for induction of systemic acquired resistance (SAR) that is triggered in response to virulent pathogens or synthetic chemicals, and is characterized by induction of pathogenesis-related proteins (PR) (Fu and Dong 2013). In addition, JA and ET plays a crucial role in resistance against necrotrophic pathogens that obtain their food by degrading plant tissue (Glazebrook 2005). ET and JA are commonly required for induced systemic resistance (ISR) that is activated upon root colonization by beneficial microbes, such as mycorrhizal fungi or plant growth-promoting rhizobacteria (PGPR), and includes the expression of non-SAR PR proteins (Zamioudis and Pieterse 2012).

The exogenous application of phytohormones, or their chemical analogs, have been shown to induce resistance against a broad spectrum of pathogens in various plant species (Beckers and Conrath 2007; Bektas and Eulgem 2014; Belhadj et al. 2008; Walters et al. 2006). For example, in soybean, application of benzothiadiazole (BTH), which is a chemical analogous to SA and an important chemical activator of SAR, can induce resistance against white mold (Dann et al. 1998) and *Phytophthora sojae* (Han et al. 2013). Also, exogenous application of β-aminobutyric acid (BABA), a non-protein amino acid, induces soybean resistance against *Aphis glycines* as it activates the expression of several defense enzymes (Zhong et al. 2014). Similarly, Sugano et al. (2013) observed enhanced resistance against *Phytophthora sojae* in soybean seedlings treated with the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), or ethephon. Exogenous application of ethephon has also induced resistance against *Botrytis cinerea* in grapevine (Belhadj et al. 2008) and *Magnaporthe oryzae* in rice (Singh et al. 2004).

The optimization of chemical elicitor application rate and timing are key factors for successful induction of plant defense responses. For example, application of salicylic acid induced resistance against the common bunt pathogen *Tilletia tritici* (Bjerk.) only when applied to 2- and 3-week-old wheat seedlings (Lu et al. 2006). Similarly, in tomato, the control of *Verticillium* wilt was more effective when ACC (ethylene precursor) was added at the time of inoculation, compared to 1 day before or after inoculation (Robison et al. 2001). Moreover, in perennial ryegrass, application of a low ethephon concentration was found to be more effective in suppressing gray leaf spot compared to higher concentrations (Rahman et al. 2014).

In a previous greenhouse study, we found that ethephon applied as soil drench enhanced resistance against SDS and induced the expression of soybean defense-related genes. However, this research was conducted under controlled environment and ethephon was applied at a single soybean growth stage before Fv infection. The objectives of this study are to 1) determine the optimum application timing of ethephon, and to 2) elucidate the effect of ethephon application on SDS development under field conditions.

## Materials and Methods

***Inoculum preparation***. *F. virguliforme* isolate (NE 305) was used as the inoculum source in all experiments. Inoculum was prepared following the procedure described by De Farias Neto et al. 2006). Sorghum seeds [*Sorghum bicolor* (L.) Moench] were soaked in water overnight to remove residuals. Three liters of sorghum seeds were then placed in autoclave bags and autoclaved at 121 C° for 1 hour, on two consecutive days. Forty plugs of Fv mycelium previously grown in 1/3 PDA media, were placed in each bag and incubated at room temperature under dark conditions for two weeks, then allowed to dry on racks at room temperature.

***Greenhouse experiment***. An experiment was conducted at the ISU greenhouse facility to test the effect of ethephon application timing on SDS development. At planting, the infested sorghum inoculum was mixed with pasteurized 2:1 sand to soil mixture at a ratio of 1:30 inoculum to sand/soil (v/v). Two soybean genotypes, Williams 82 (susceptible to SDS) and MN1606 (resistant to SDS), were used in all experiments. Four seeds were sown 1

cm below the soil surface in square plastic pots (12 cm), and then thinned to two seedlings after germination. After planting, ethephon (0.1mM) was applied at different soybean growth stages according to the following five treatments: 1) soil drench at planning (VP), 2) VP + emergence (VE), 3) VP + foliar sprayed at unifoliate stage (VC), 4) VP + foliar sprayed at V1-V2, and 5) untreated control. For the soil drench, each pot received approximately 15 ml of ethephon. For the foliar applications, ethephon was applied on the upper and lower leaf surface until runoff using a hand sprayer. All pots were incubated on a greenhouse bench at 24°C under a 16-h photoperiod, watered once a day, and fertilized weekly. The experiment was setup as a 2 x 5 x 2 factorial design with two *Fv* inoculation levels (inoculated and non-inoculated), five ethephon treatments (VP, VP+VE, VP+VC, VP+V1-V2, and untreated control), and two soybean cultivars (Williams 82 and MN1606). Plants were arranged according to a randomized complete block design with 7 replicate pots per treatment combination. Fourteen plants (two per each of seven replicate pots) were non-destructively rated for SDS foliar symptoms at 28, 35, 42, and 49 days after planting (DAP) and root rot was rated on plants destructively sampled at 49 DAP. Foliar SDS severity was rated visually as the percent leaf area showing chlorosis and/or necrosis, and root rot was rated as the percent root area showing brown or black discoloration. Number of pods, defined as visible pods in any node were counted in all plants at 49 DAF. Root and shoot were destructively sampled at 49 DAF for dry weight by rinsing in tap water to remove soil particles then incubated in oven at 70°C for 2 days.

***Field experiment.*** Field trials were established in 2015 and 2016 at the ISU Hinds Research Farm in Ames, IA and at a grower farm, in Roland, IA, to examine the effect of

ethephon treatment on SDS development under field conditions. Both fields were under a corn-soybean rotation and had a previous history of SDS. At the Hinds Farm, each row was artificially infested with Fv by incorporating 1.5 g of infested sorghum grain per 30 cm of row, at the time of plating. No artificial inoculation was used at the Roland farm. Tillage was done prior to planting in both locations, and post-emergence weed was controlled using glyphosate herbicide at the recommended rates. Planting dates in 2015 were 29 April at Hinds and 13 May at Roland farms, respectively.

Two-by-four factorial experiment, consisting of soybean genotype with two levels: AG2933, glyphosate tolerant, maturity group 2.9, SDS resistance score of 5 on a 1-9 scale where 1=excellent and 9= poor, and P22T41R2, glyphosate tolerant, maturity group 2.2, and SDS resistance score of 3 on a 1-9 scale where 1=poor and 9=excellent, and ethephon treatment with four levels: untreated control, soil drench at planting (in-furrow), at emergence (VE), and foliar spray at V1-V2 were arranged in a randomized complete block design, with four blocks. For ethephon application, approximately 500 ml of 0.1 mM concentration was dissolved in deionized water then applied either as soil drench at the following growth stages, i) planting (in-furrow), ii) post-emergence (VE), or as foliar spray at iii) V1-V2, to the middle two rows using backpack sprayer, untreated plots were used as control. In 2016, planting dates were 6 and 16 May at Hinds and Roland locations, respectively. The experiment followed a 2 x 5 factorial design due to the addition of two treatments that used a higher rate of ethephon (1mM) and were applied at planting (in-furrow), and post-emergence (VE). Each plot was an experimental unit and consisted of four rows, with 5.3 m in length and inter-row spacing of 76 cm.

***Disease Assessments.*** SDS foliar incidence (DI) and severity (DS) were visually evaluated in the middle two rows of each plot starting at disease onset. DI was estimated as the percent of symptomatic SDS plants, and DS was estimated on 0-to-9 scale in which 0= no disease symptoms, and 9= premature defoliation (Gibson et al. 1994). Foliar disease index (DX) was calculated using the following formula: DX= (DI x DS)/9. In 2015, DX was evaluated starting at R3 growth stages for six and five scoring dates at one-week interval in the Hinds and Roland locations, respectively. Area under disease progress curve (AUDPC) was calculated for DX as described by (Simko and Piepho 2012). In 2016, disease ratings were conducted at R6 growth stage for one and three weekly ratings, at Hinds and Roland locations, respectively.

***Plant population and yield data collection.*** Plant stand was determined at an early vegetative growth stage (V2-V3) by counting the total number of live plants in a 3-m length of row in the middle two rows of each plot. At growth stage V5-V6, shoot length of 3 or 5 plants were measured from the ground to the growing point. Yield data were collected at maturity by harvesting the center two rows with a small-plot combine, and grain yield was adjusted to 13% moisture.

**Data analysis.** For the greenhouse experiment, ANOVA was performed using the PROC GLIMMIX procedure of SAS version 9.4 (SAS Institute, Cary, NC) to determine the main effect of ethephon treatment on root rot, SDS foliar disease severity, plant dry matter, and number of pods. Orthogonal contrasts were used for SDS foliar and root rot severity, and soybean growth parameters to compare ethephon treatments at VP+VE and VP+VC with the

untreated control. For the field experiment, ANOVA was performed to determine the main effect of ethephon treatment on DX, AUDPC, plant population, shoot length, and grain yield. Orthogonal contrasts were used for DX, AUDPC, and soybean growth parameters to compare ethephon treatments to the untreated control. Ethephon treatment, and cultivar were treated as fixed factors and blocks as random factor. Fisher's protected least significant difference test was used to compare the differences in treatment means at the $P = 0.05$ significance level.

## Results

### Greenhouse experiment.

There was significant main effect of ethephon treatment on SDS foliar severity at 28 and 35 DAP. Also, there was cultivar main effect on SDS foliar severity at all rating time points tested. No cultivar by treatment interaction was observed in most time points; therefore, cultivars were combined to present the treatment effect.

***Effect of ethephon treatment on SDS foliar severity***. All ethephon treatments resulted in a significant ($P<0.05$) reduction of foliar SDS severity at 28 and 35 DAP rating time points compared to the untreated control, however, no significant effect was observed at 42 and 49 DAP (Fig. 1)**.** No significant differences in SDS foliar symptoms were observed among the different ethephon treatments, ie. the reduction in SDS severity was not affected by ethephon application frequency. Plants that received one application at planting exhibited no difference in disease severity compared to plants that received two applications; one at planting and another one at VP, VE, VC, or V1-V2 growth stages (Fig. 1).

***Effect of ethephon treatment on root rot.*** There were no significant main effects of ethephon treatment and cultivar on root rot severity. There was also a significant interaction between treatment and cultivar, therefore; data for the treatment effect was presented separately by cultivar. Orthogonal contrast revealed that plants receiving ethephon application at VP+VE growth stages had less root rot severity compared to the water treated control ($P<0.05$) in cultivar MN1606, but no significant difference was observed in the susceptible cultivar (Fig. 2).

***Effect of ethephon treatment on soybean growth***. The main effect of ethephon treatment was not significant for shoot dry weight, root dry weight, and pod number. There was significant main effect of cultivar ($P<0.001$) for these variables, with the resistant cultivar MN1606 showing greater shoot dry weight and pod number compared to the susceptible cultivar Williams 82. There was no significant interaction between cultivar and treatment; therefore cultivars were combined to present the effect of ethephon treatment (Table 1). Orthogonal contrast revealed that root dry weight was greater in plants treated with ethephon at VP+VC growth stage showed higher shoot and compared to the control ($P<0.05$), and plants treated at VP+VE growth stages showed only higher root dry weight compared to the control ($P<0.01$) (Table 1).

**Field experiment**

***Artificially infested field plots.*** At the Hinds farm field trial in 2015, no significant main effect of ethephon treatment was observed on SDS foliar severity at any rating time.

The main effect of cultivar was significant ($P<0.001$) at all time points, with cultivar AG2933 showing less SDS symptoms compared to cultivar P22T41R2 (Fig. 3). No significant interaction between treatment and cultivar was observed, therefore, cultivars were combined to present the treatment effect. In 2015, orthogonal contrast revealed that plots drenched with ethephon at VE growth stage had less SDS foliar severity ($P<0.03$) compared to the untreated control, at all rating times. In addition, AUDPC for disease index (DX) was 57% lower in plots exposed to ethephon treatment at VE growth stage compared to untreated plots (Table 2). In 2016, due to the low disease pressure, no treatment differences were observed in either location. However, the same numerical pattern was observed, where plots treated with ethephon at VE growth stage had the lowest SDS severity.

***Naturally infested field trial.*** At the Roland field trial, there were no significant main effects of ethephon treatment and cultivar on SDS foliar disease severity at any rating time point. Also, no significant interaction between treatment and cultivar was observed, therefor, cultivars were combined to present the effect of ethephon treatment.

In 2015, at R6 growth stage, the orthogonal contrast revealed that there was a 75% reduction in SDS foliar severity in plots received the in-furrow treatment compared to untreated plots. However, no significant differences among ethephon treatments were observed in other rating times (Fig. 4B). In additions, the AUDPC for DX showed no significant differences among ethephon treatments (Table 2). In 2016, as a result of the low disease pressure, no treatment effect was detected in either cultivar.

***Yield response to ethephon treatment.*** In both locations and both years, there were no significant main effects of ethephon or cultivar on soybean yield, and no significant interaction between ethephon treatment and cultivar. Therefore, yield data are presented for each ethephon treatment averaged over cultivar (Table 3). Despite the lack of significant differences among treatments in 2015, yield in plots treated with ethephon application at VE growth stage was numerically greater by 15% and 18% at Hinds and Roland, respectively, compared to the untreated control. In 2016, yield values were almost the same among ethephon treatments in both locations Table 3).

## Discussion

The present study indicated that ethephon application influences SDS development under greenhouse and field conditions, and that the overall effect on SDS development depends on application timing. For instance, under field conditions, ethephon application at VE growth stage significantly reduced SDS severity compared to the untreated control, whereas application at V1-V2 growth stage had no effect. Furthermore, under greenhouse conditions, all ethephon treatments resulted in reduction of SDS foliar symptoms compared to the untreated control. These findings support our earlier research showing that soil drench application of ethephon induces resistance against SDS, probably due to induction of soybean defense response genes (Abdelsamad et al. unpublished data).

Our findings on reduction of SDS severity due to application of ethephon are also consistent with previous reports in other pathosystems. For instance, in soybeans, a field experiment showed that application of ethephon in midseason reduced incidence of

seedborne fungi such as *Phomopsis* spp. and *Cercospora kikuchii* (Abney and Ploper 1991). Sugano et al. (2013) also showed that application of ethephon 24 hours before inoculation induced soybean resistance to *Phytopthora sojae* due to induction of defense-related genes. The mechanisms underlying the reduction of SDS in response to ethephon treatment was not investigated in this study. However, previous studies showed that exogenous application of ethephon resulted in ethylene accumulation and enhanced resistance against several pathosystems, including charcoal rot in *Medicago truncatula* (Gaige et al. 2010), *P. sojae* in soybean (Sugano et al. 2013), *P. capsici* in Habanero pepper (Nunez-Pastrana et al. 2011), and *Erysiphe necator* in grapevine (Belhadj et al. 2008), probably due to accumulation of pathogenesis-related proteins and antimicrobial compounds such as phytoalexins. Previously in our lab, a greenhouse experiment revealed that soil drench application of ethephon 24 hours before and after Fv inoculation induced the expression of pathogenesis-related proteins and other defense-related genes (Abdelsamad et al. unpublished data).

Another possible explanation for reduction of SDS in response to ethephon application could be due to the toxic effect of ethephon on Fv growth and development. Ethephon has been shown to inhibit mycelial growth of several pathogens like *Botryosphaeria* spp. and *P. capsici* (Li et al. 2014; Nunez-Pastrana et al. 2011). Furthermore, in the soybean-Fv pathosystem, a previous study in our lab showed that ethephon application induces the expression of defense-related genes and enhance resistance against SDS (Abdelsamad et al. unpublished data). Although not tested in this study, is it possible that the accumulation of defense-related genes and/or inhibition of Fv mycelium growth in response to ethephon treatment might explain the reduction of SDS severity in ethephon treated plants compared to control.

This research demonstrates that ethephon application timing is an important factor to consider if used to manage SDS under field conditions. For instance, early application of ethephon at planting or after seed germination reduced SDS development under field conditions, whereas application at V1-V2 growth stage showed no control of the disease. One possible explanation for this differential effect could be that the method of application, soil drench vs foliar spray, could impact effectiveness. It is also possible that waiting until this stage to apply ethephon might be late to control the disease, as pathogen infection could have already established in roots. Since ethephon was applied as a soil drench at early growth stages, and as a foliar spray at V1-V2 growth stage, it is not possible to conclude if the lack of effectiveness at V1-V2 stage was due to the later stage of development or due to the foliar spray. In the greenhouse experiment, a single ethephon application at planting was sufficient to control the disease compared to an application at planting followed by additional applications. Similarly, Singh et al (2004) found that multiple applications of ethephon can control rice blast disease caused by *Magnaporthe grisea* to the same extent as a single ethephon application.

The results from the greenhouse and field experiments showed that the effects of ethephon application were more evident in susceptible cultivars compared to the resistant cultivars. This might be due to the preexistence of strong defense mechanisms in the resistant cultivars, making it difficult for ethephon application to make any further enhancement in disease resistance. Similarly, with different chemical inducers, Dann et al. (1998) showed that application of benzothiadiazole (BTH) or 2,6-dichloroisonicotinic acid (INA) controlled white mold caused by *Sclerotinia sclerotiorum*, but that the effect was less pronounced in the

resistant cultivars compared to the susceptible one. It is not possible to conclude from this study if ethephon application on resistant cultivars would show any significant suppression under conditions of higher disease pressure. Another limitation of our study is that we did not investigate the effect of ethephon treatment on *Fv* population in soil or roots to have a better understanding how ethephon affects *Fv* survival and pathogenicity.

In summary, this study demonstrated that ethephon applied as a soil drench can reduce SDS foliar symptoms under greenhouse and field conditions. Furthermore, under field conditions, ethephon application has no negative effect on soybean growth and development as indicated by stand counts, shoot length, and grain yield. Future work should validate the findings reported here using large-scale field trials with multiple locations and other plant defense activators to determine if the incorporation of this plant defense activator could be a useful management strategy to control SDS under field conditions.

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**Table 1.** Effect of ethephon treatment[a] on soybean root dry weight (RDW), shoot dry weight (SDW), and number of pods in greenhouse conditions.

| | Growth parameters [b] | | |
|---|---|---|---|
| Treatment | RDW (g) | SDW (g) | Number of pods |
| Control | 0.51 | 1.60 | 1.60 |
| VP | 0.68 | 1.57 | 0.92 |
| VE+VP | 0.78 | 1.99 | 1.53 |
| VC+VP | 0.86 | 2.15 | 1.20 |
| V1-V2+VP | 0.70 | 1.92 | 1.35 |
| P -value | 0.08 | 0.25 | 0.42 |
| | | | |
| Contrasts (*P*- value)[c] | | | |
| Control vs. VP+VE | 0.03 | 0.19 | 0.85 |
| Control vs. VP+VC | 0.01 | 0.07 | 0.30 |

[a] Approximately 15 ml of ethephon was applied as a soil drench at VP, VE, and VC growth stages; at V1-V2 growth stage ethephon was foliar sprayed until run off.
[b] Data represent the mean of 14 replicate plants (combination of seven replications for each of two cultivars) for RDW, SDW, and number of pods
[c] Orthogonal contrasts were used to compare ethephon applications at VP+VE and VP+VC to the untreated control

**Table 2.** Effect of ethephon application on sudden death syndrome foliar symptoms expressed as the area under disease progress curve (AUDPC) of disease index at Hinds and Roland locations for year 2015

| | AUDPC[a] | |
|---|---|---|
| Location[b] | Hinds | Roland |
| Treatment[c] | | |
| Control | 114.14 | 58.62 |
| VP (In-Furrow) | 77.46 | 18.68 |
| VE | 48.33 | 61.50 |
| V1-V2 | 102.24 | 36.41 |
| *P*-value | 0.13 | 0.18 |
| | | |
| Contrast (*P*-value)[d] | | |
| Control Vs. VE | 0.02 | 0.89 |
| Control Vs. VP | 0.20 | 0.07 |

[a] Data represents the mean area under disease progress curve (AUDPC) of SDS foliar disease index for the average of two cultivars, based on six, and five weekly visual disease ratings at Hinds and Roland locations, respectively. Disease index was calculated using the following formula; (Disease incidence*Disease severity)/9.

[b] Hinds farm plots were artificially inoculated with *Fusarium virguliforme* isolate NE 305, and Roland farm plots were naturally infested.

[c] Ethephon treatment: control = untreated, VP = in-furrow application at planting, VE = soil drench application after seed germination, V1-V2 = foliar spray application.

[d] Orthogonal contrasts were used to compare ethephon treatment at VP and VE with the untreated control

**Table 3.** The effect of ethephon application on soybean yield in bushels per acre collected at Hinds and Roland locations, in Iowa, in 2015 and 2016.

| | Yield (Kg/ha)[a] | | | |
|---|---|---|---|---|
| Location[b] | Hinds | | Roland | |
| Year | 2015 | 2016 | 2015 | 2016 |
| Treatment[c] | | | | |
| Control | 3238 | 4297 | 2905 | 4531 |
| VP | 3546 | 4355 | 3377 | 4404 |
| VE | 3845 | 4310 | 3538 | 4194 |
| V1-V2 | 3139 | 4106 | 3338 | 4045 |
| *P-value* | 0.270 | 0.390 | 0.730 | 0.350 |
| LSD | 10.2 | 4.5 | 16.2 | 8.5 |

[a] Data representing mean yield (Kg/ha) pooled for soybean cultivars AG2933 and P22T41R2. Yield data were collected at maturity, and grain yield was adjusted to 13% moisture.

[b] Hinds farm plots were artificially inoculated with *Fusarium virguliforme* isolate NE 305, and Roland farm plots were naturally infested with this pathogen.

[c] Ethephon treatments: control = untreated, VP = in-furrow application at planting, VE = soil drench application after seed germination, V1-V2 = foliar spray application.

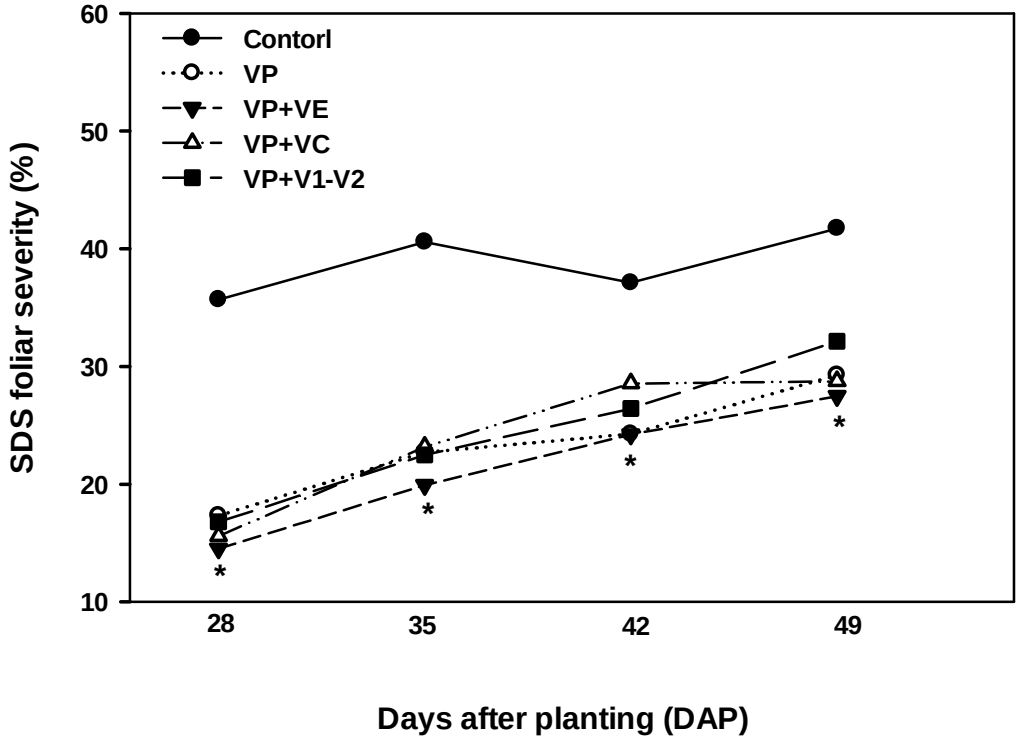


**Fig. 1.** Effects of timing and frequency of ethephon (0.1mM) application at different soybean growth stages on SDS foliar disease severity in soybean seedlings at 28, 35, 42, and 49 days after planting (DAP) under greenhouse conditions. Control = untreated, VP= ethephon application at planting, VP+VE= one application at planting and another application at seed emergence, VP+VC= one application at planting and another application at unifoliate growth stage, VP+V1-V2= one application at planting and another application at first or second trifoliate growth stage. Each dot represents the average percentage of foliar disease severity of fourteen replications (2 cultivars x 7 replications). Points followed by asterisk are significantly different ($P$<0.05) from control based on orthogonal contrast.

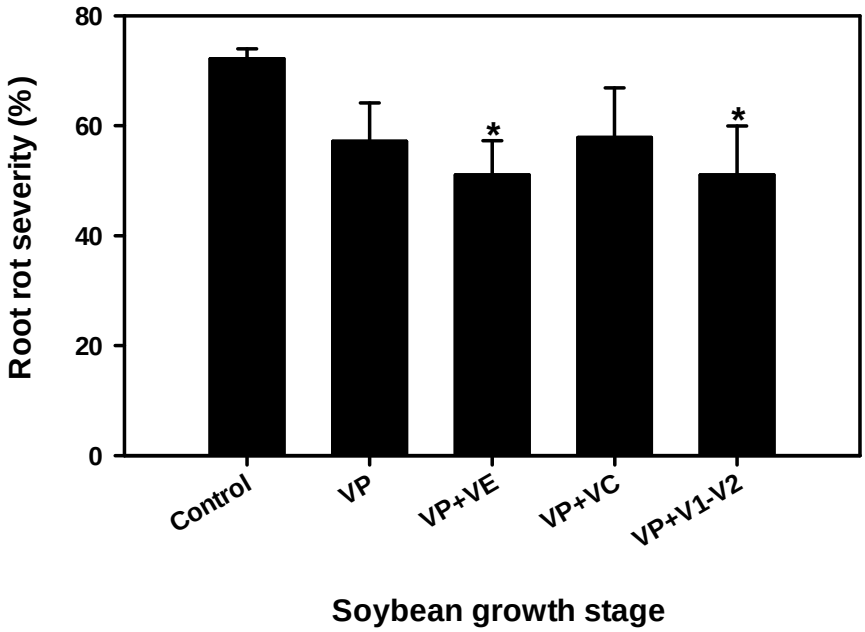


**Fig. 2** Effects of application timing and frequency of ethephon 0.1mM at different soybean growth stages on SDS foliar disease severity in resistant cultivar MN1606 at 49 days after planting (DAP) under greenhouse conditions. Control = untreated, VP= ethephon application at planting, VP+VE= one application at planting and another application at seed emergence, VP+VC= one application at planting and another application at unifoliate growth stage, VP+V1-V2= one application at planting and another application at first or second trifoliate growth stage. Each bar represents the average percentage of root rot severity of seven replications. Error bar represents the standard error of the mean. Bars followed by asterisk are significantly different ($P$<0.05) from control based on orthogonal contrast.

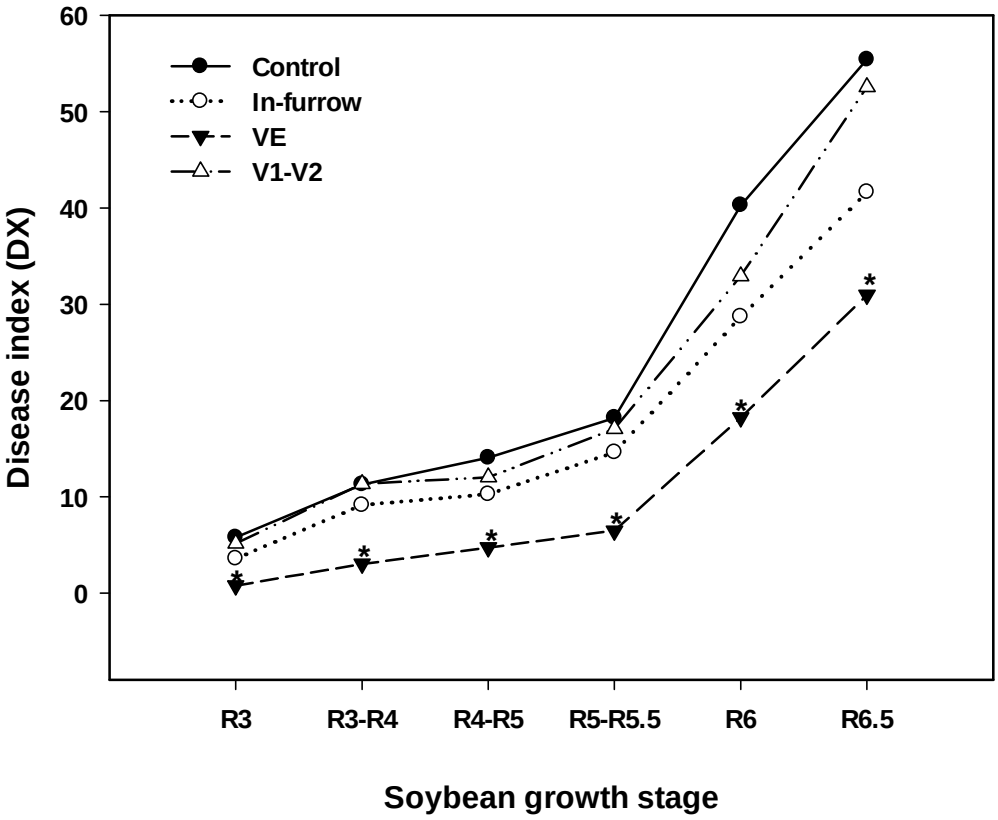


**Fig. 3.** Effects of ethephon (0.1mM) application at different growth stages on SDS foliar disease severity at Hinds location (artificially inoculated with *Fusarium virguliforme*) in 2015. Control = untreated, In-furrow= ethephon application at planting as soil drench, VE= ethephon application after seed emergence as soil drench, V1-V2= ethephon application at first or second trifoliate growth stage as foliar spray. Each dot represents the average percentage of foliar disease index (DX) of four replications. Mean followed by asterisk are significantly different ($P$<0.05) from control based on orthogonal contrast.

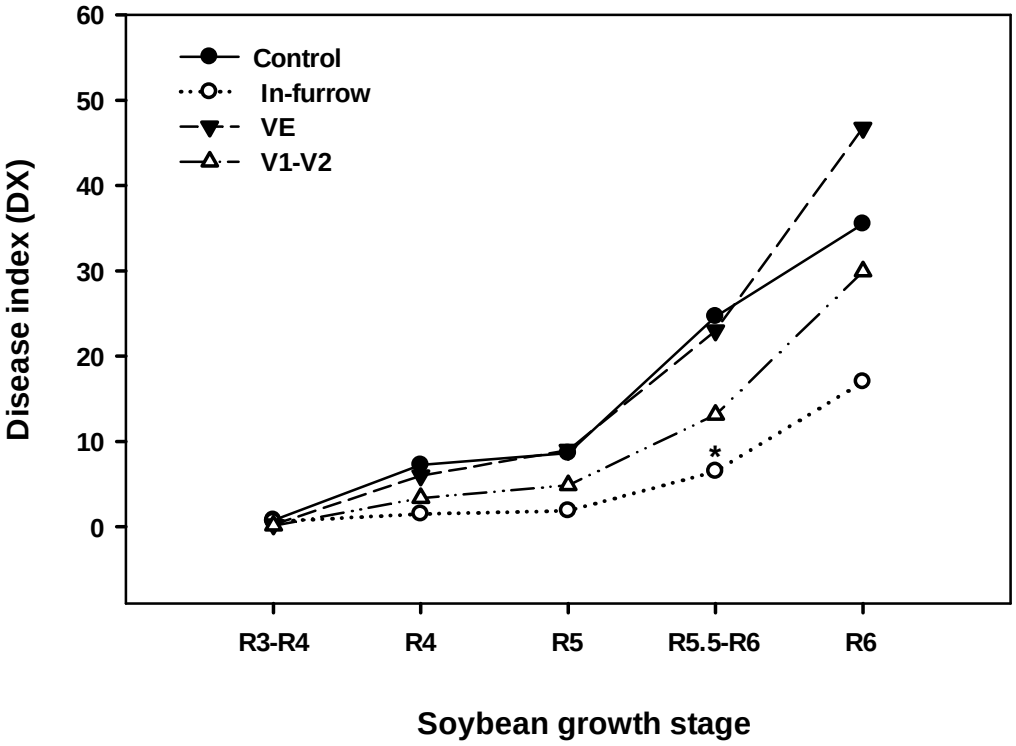


**Fig. 4.** Effects of ethephon (0.1mM) application at different growth stages on SDS foliar disease severity at Roland location (naturally infested with *Fusarium virguliforme*) in 2015. Control = untreated, In-furrow= ethephon application at planting as soil drench, VE= ethephon application after seed emergence as soil drench, V1-V2= ethephon application at first or second trifoliate growth stage as foliar spray. Each dot represents the average percentage of foliar disease index (DX) of four replications. Mean followed by asterisk are significantly different ($P$<0.05) from control based on orthogonal contrast.

# CHAPTER 5.

# GENERAL CONCLUSIONS

Major soybean sudden death syndrome outbreaks have coincided with years of extreme flooding, such as 1993, 2008, and 2010, but there is no information about how and why excessive soil moisture is associated with severe SDS. Flooding conditions cause a decrease in oxygen levels and build-up of carbon dioxide and ethylene hormone in the root zone (Fig. 1). The work presented in this dissertation investigated the effect of flooding duration, anaerobic conditions, and the ethylene hormone on the development of soybean sudden death syndrome caused by *Fusarium virguliforme*.

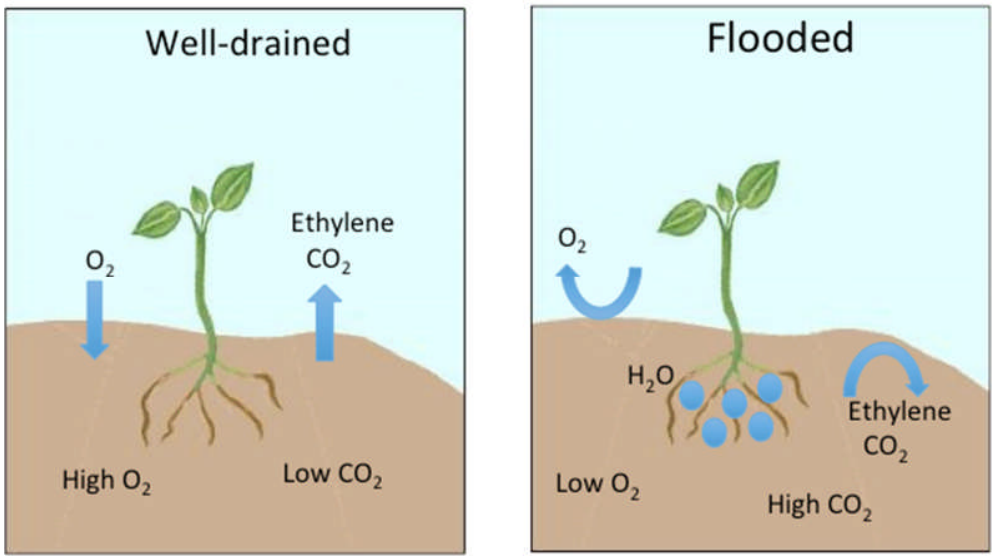


**Fig. 1.** Comparison of gas exchange in well drained and flooded soil environments

Our studies showed that flooding and the accompanied anaerobic conditions influences SDS disease severity, *Fv* population density in soil, soybean defense-related genes, and *Fv* virulence genes under greenhouse and hydroponic conditions, although the overall effect on SDS depends on duration of the flooding period and the oxygen level. For instance, 3 days of continuous flooding and repeated 8h periods of flooding generally did not affect *Fv* population in soil, and the oxygen levels representative of those two flooding treatments had no effect on *Fv* virulence genes. This might explain the increase in SDS development that was observed under short-term flooding treatment. On the other hand, the reduction of SDS severity under long-term flooding periods for 5- and 7-days could be due to the reduced *Fv* population in soil under those conditions. Moreover, the oxygen levels representative of long-term flooding regimes were shown to generally decrease the expression of *Fv* virulence genes tested in our study (Fig. 2).

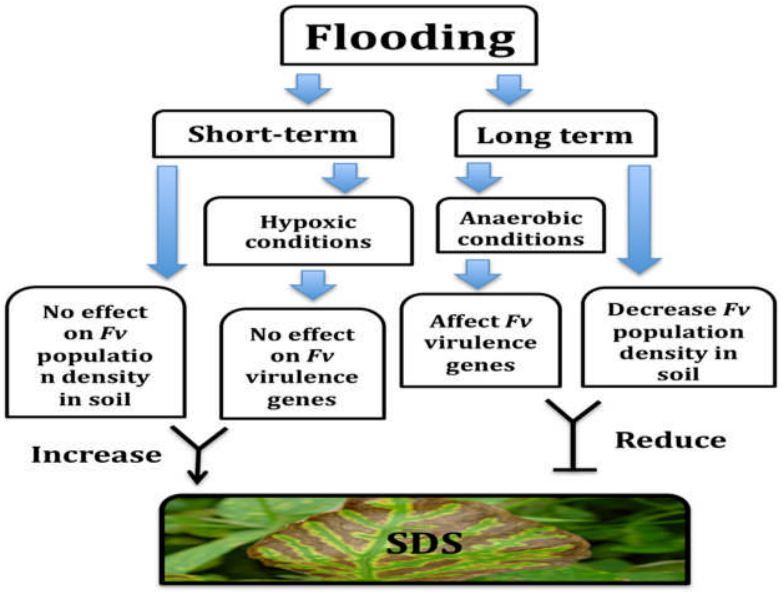


**Fig. 2.** A proposed model for the effect of flooding and oxygen levels on SDS development

One of the other changes that happens during flooding conditions is the accumulation of ethylene hormone in plants. In our study we investigated the role of the ethylene hormone on soybean response to SDS using pharmaceutical approach. Our study showed that soil drench application of ethephon to soybean seedlings induced the expression of genes related to ethylene biosynthesis and defense response in soybean roots. In additions, we also found a direct inhibitory effect of ethephon application on *Fv* growth and sporulation *in-vitro*. Taken together, this might explain the reduction of SDS foliar symptoms in seedlings exposed to ethephon treatment compared to untreated control plants. On the contrary, an increase on SDS foliar symptoms was observed in seedlings exposed to 1-MCP which had little or no effect on the expression of ethylene biosynthesis genes and soybean defense-related genes (Fig. 3). Collectively, these results suggest that ethylene signaling pathway might be important in resistance against *Fv*.

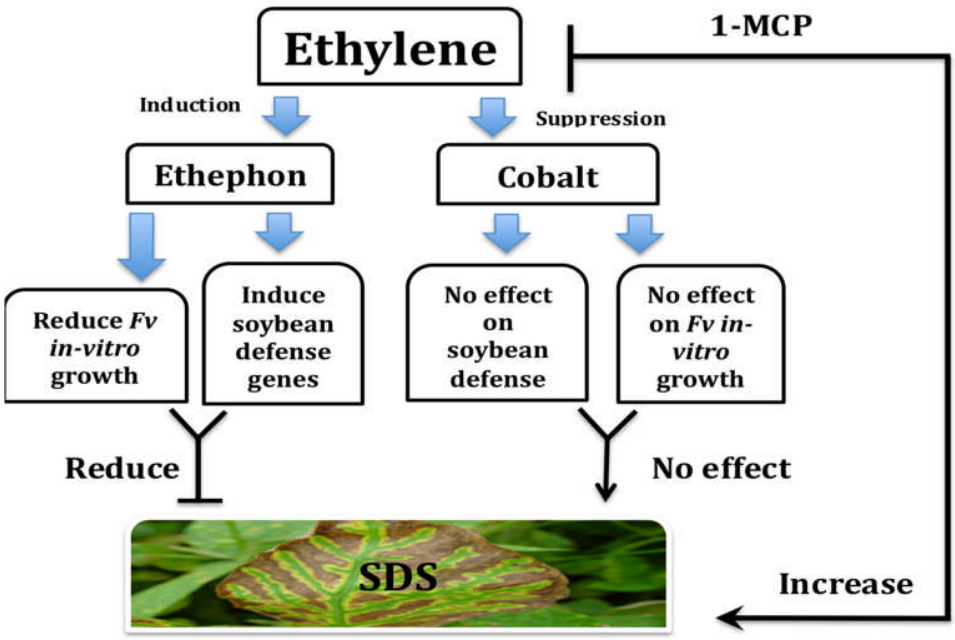


**Fig. 3.** A proposed model for the effect of ethylene manipulation on SDS development

Finally, we found that ethephon application influences SDS development under greenhouse and field conditions, and that the overall effect on SDS development depends on application timing. Under field conditions, ethephon application at VE growth stage significantly reduced SDS severity compared to the untreated control, whereas application at V1-V2 growth stage had no effect. Furthermore, under greenhouse conditions, all ethephon treatments resulted in reduction of SDS foliar symptoms compared to the untreated control. Further research is needed to validate the findings reported here using large-scale field trials with multiple location to determine if the incorporation of this type of plant defense activator could be a useful management strategy to control SDS under field conditions.

To the best of our knowledge, the research presented in this dissertation is the first to investigate the effects of flooding, anaerobic conditions, and ethylene hormone on the soybean-*Fv* interaction and the development of SDS. The findings from this study will be useful for researchers to better understand the impact of abiotic stresses such as flooding and anaerobic conditions on disease development. This study also starts to decipher the role of the ethylene hormone on SDS development, and opened an avenue for SDS researchers to further investigate the use of plant activators as a management strategy to control SDS.